Genetic variation in energy metabolism: impact of the B2-adrenoceptor polymorphism Gly16Arg

Genetic variation in energy metabolism

Impact of the β_2-adrenoceptor polymorphism Gly16Arg

The study presented in this thesis was performed within the Nutrition and Toxicology Research Institute Maastricht (NUTRIM) which participates in the Graduate School VLAG (Food Technology, Agrobiotechnology, Nutrition and Health Sciences)

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1

General introduction

Obesity

Epidemiology of obesity and contributing factors

There are several definitions of overweight and obesity. The most practical current definitions are the ones of the World Health Organization (WHO) with overweight defined as a body mass index (BMI) between 25-30 kg/m^2 and obesity defined as a BMI of 30 kg/m^2 or greater [1].

The prevalence of obesity has been increasing dramatically in western societies during the last decades of the 20th century and in non-western societies more recently. In 2002, about 28% of adult men and 34% of adult women in the United States was obese (Center for Disease Control and Prevention, USA), and in 2005 nearly 10% of the Dutch men and 12% of the Dutch women was obese (CBS, The Netherlands) according to the World Health Organization definition.

Obesity occurs when there is an imbalance between energy intake and energy expenditure leading to an energy surplus in the body. The energy surplus is mainly stored in the adipose tissue. Despite this simple description, the phenomenon obesity is complex because various factors influence energy intake and energy expenditure.

Obesity is determined by genetic and environmental factors that are incompletely understood. Several twin studies have shown a strong genetic component of BMI [2-4] accounting for up to 70% of variance in BMI. With regards to the genetic origin of obesity, it has been suggested that some single gene defects may predispose patients with extreme obesity, whereas polygenic effects may be involved in the range from thin to fat [5]. Genetic predispositions have received great attention, although the underlying cause of the dramatic increase in obesity prevalence in industrialized countries within the last few decades is most likely due to changes in environmental factors, such as changes in energy intake from dietary fat and alcohol, changes in fiber intake, and a decrease in level of physical activity [6]. The susceptibility for these environmental factors however, may vary from person to person because of differences in genetic background [7].

The genetic variation, described as polymorphisms, of a large number of genes can influence the sensitivity for obesity and other multi-factorial diseases. A search in PubMed with the searchkeys "obesity" and "polymorphism" results in 1501 hits (19-4-2006). Already for years Obesity Research publishes yearly an update of the human obesity map were all known genes linked to obesity are listed [8]. From this gene map it becomes clear that, although several candidate genes are known, the explained variance in BMI is still very low.

Sympathetic nervous system

Energy expenditure and the sympathetic nervous system

The sympathetic nervous system (SNS) plays an important role in the regulation of energy expenditure. SNS activity is stimulated in response to food digestion and physical exercise, but it can also be triggered by cold exposure or pathogenic stimuli [9]. In response to these stimuli, sympathetic nerve fibers release noradrenaline into the synaptic cleft, where it functions as a neurotransmitter. Part of the noradrenaline spills over into the blood, where it acts as a hormone. Furthermore in response to these stimuli, the adrenal medulla excretes adrenaline into the blood. The SNS exerts its effects through noradrenaline and adrenaline binding to α_1-, α_2-, β_1-, β_2-, and β_3-adrenoceptors on target cells.
In humans, total energy expenditure represents the sum of the obligatory use of ATP to sustain life, to generate muscular activity, and to respond to the surrounding environment. The main component, which is obligatory energy expenditure or obligatory thermogenesis, is the basal metabolic rate that has a significant thyroid hormone-regulated component. This basic cost is attributed to nucleic acid synthesis and substrate cycling (30%-35%), protein turnover (20%-25%), sodium and potassium pumping (20%-25%), gluconeogenesis (7%), calcium pumping (5%), the actomyosin ATP-ase (5%), and ureagenesis (2.5%)[10]. Also included in the obligatory component of thermogenesis is the thermic effect of food, the effort required to digest and absorb nutrients. Measured thermic effects of nutrients are 0%-3% for fat, 5%-10% for carbohydrates, and 20%-30% for proteins [11]. Energy expenditure associated with physical exertion occurs mainly within the skeletal muscle, with a small but non-neglectible role for the liver. Facultative thermogenesis is superimposed on obligatory thermogenesis and can be rapidly switched on and rapidly suppressed by the nervous system. Facultative thermogenesis is important in both thermal balance, in which control of thermoregulatory thermogenesis (shivering in muscle, nonshivering in rat in brown adipose tissue (BAT)) balances heat loss mechanisms, and in energy balance, in which control of facultative thermogenesis (exercise-induced in muscle, diet-induced thermogenesis (DIT) in BAT) balances energy intake [12].

The relationship between daily energy expenditure and activity of the sympathetic nervous system has not been well studied. In two studies [13,14] a positive correlation between 24 hour energy expenditure, adjusted for body size and body composition, and measures of sympathetic nervous system have

been found during a stay in a respiration chamber, but in another study no significant relationship was present [15]. Furthermore, β-adrenoceptor blockade did not reduce 24 hour energy expenditure significantly [16,17]. Astrup and MacDonald calculated that the differences in energy expenditure between subjects with high and low sympathetic nervous system activity was approximately 750 kJ day^{-1}. These data suggest that interindividual differences in sympathetic nervous system activity induce variations in energy expenditure that may be relevant to energy balance. The influence of the sympathetic nervous system activity on resting metabolic rate is relatively small. Administration of the β_1- and β_2-adrenoceptor blocking agent propanolol reduces resting metabolic rate by 3-4% [13-20]. However after a meal, energy expenditure, as well as sympathetic nervous system activity, rise. The increase in energy expenditure is positively correlated with the increase in appearance rate of noradrenaline [21]. The quantitative contribution of the sympathetic nervous system to the thermic effect of food (on average 30-40%) depends strongly on the size and the nutrient composition of the meal, particularly on the carbohydrate content and partly on protein content of the meal which is positively correlated with the sympathetic nervous system response [22]. Increase in energy expenditure as a result of exercise cannot be reduced by β-adrenoceptor blockade and the increased energy expenditure after exercise also does not appear to be related to sympathetic nervous system activation, since it cannot be blocked by β-adrenoceptor antagonists [23].
During the infusion of noradrenaline [24,25], adrenaline [26,27] or isoprenaline [20] (non-selective β-adrenoceptor agonist), thermogenesis increases significantly. The role of the individual adrenoceptor subtypes in thermogenesis is not completely known. α-Adrenergic stimulation does not affect whole body thermogenesis [20,28], whereas selective β_1- [29,30] or β_2-adrenergic stimulation [20,29,31] increases thermogenesis.

Lipolysis and the sympathetic nervous system

The role of the SNS in the control of lipolysis has been investigated in many ways in the past century and has been thoroughly reviewed [32]. Briefly, studies showed that denervation of white fat depots lead to tissue hypertrophy [33] and, reciprocally, that electrical stimulations of these white adipose tissue nerves led to fatty acid release [34,35]. The response elicited by these electrical stimulations were blocked by manipulations that prevented noradrenaline release or noradrenaline binding to β-adrenoceptors and were potentiated by α-adrenergic blockers and inhibitors of phosphodiesterases [34,36].

The α-adrenoceptors and the β-adrenoceptors are the recipients of these catecholamine signals. They are members of the large family of G-protein coupled receptors that are integral plasma membrane proteins. There are three subtypes of β-adrenoceptors (β_1-adrenoceptor, β_2-adrenoceptor, and β_3-adrenoceptor) all of which are expressed in white and brown (rat) adipocytes [37,38]. However the relative proportions of these subtypes vary between species, fat depots, and metabolic status [39]. In humans the control of lipolysis by the β-adrenoceptors is principally initiated by the sequential activation of adenylyl cyclase and cAMP-dependent protein kinase (PKA), ultimately culminating in the phosphorylation of hormone-sensitive lipase (HSL) and perilipin A [40-42].
In addition to the β-adrenoceptor stimulation of lipolysis, catecholamines can also be antilipolytic by stimulating the α_2-adrenoceptor. This reulsts in inhibition of cAMP production through a Gi-protein coupled decrease in adenylyl cyclase activity. The balance between the number of the β-adrenoceptor and α-adrenoceptor can thus determine the relative efficacy of catecholamines for triglyceride hydrolysis. In that respect, there is some evidence from experimental studies in animals and humans that a shift to a higher α_2/β ratio can contribute to obesity and net lipid storage [43].

The role of the sympathetic nervous system in human obesity

Impaired SNS activity leads to a decrease in energy expenditure and may play a role in the etiology of obesity, whereas stimulation of the SNS leads to an increase in energy expenditure and may be a useful tool for the treatment and prevention of obesity. Therefore, the SNS is certainly one of the first target physiological functions, which has to be studied in relation to obesity.
To study the sympathetic nervous system activity in obesity, different methodological approaches have been applied. In many studies plasma or urine catecholamine levels have been measured. MacDonald [44] reviewed the available data in 1995. Noradrenaline concentrations were lower in obese than in lean subjects in 14 studies, equal in 21 studies and higher in obese than lean subjects in 11 studies. Microneurographic recordings of the peroneal nerve, innervating the skeletal muscle circulation, show a strong positive relationship with body weight, body mass index or percentage body fat [45-48]. Regional noradrenaline spillover measurements suggest that sympathetic nervous system activity in obesity is normal for whole body (also for adrenaline spillover), but increased in the kidneys and reduced in the heart [49,50]. In contrast, whole body noradrenaline spillover was positively correlated with body fatness in another study [51]. Analysis of heart rate variability in obese subjects suggests that they have increased sympathetic nervous system

activity [52]. There is some evidence that the response of the sympathetic nervous system to various physiological stimuli (underfeeding, hyperinsulinaemia, cold exposure) is blunted in those who are obese [45,53].
So in general, the data point to a normal to increased level of sympathetic nervous system activity in most tissues, while responsiveness may be blunted, in established obesity. A high basal level of sympathetic nervous system activity in obesity may be associated with the development of co-morbidities such as insulin resistance, dyslipidemia and hypertension.
In obese subjects, the increase in energy expenditure has been found to be impaired during noradrenaline [54] or isoprenaline [55] infusion, although others found no decreased noradrenaline [26], adrenaline [56] or isoprenaline-induced [31] thermogenic response. An impaired thermogenic response due to decreased SNS activity will make individuals more prone to a positive energy balance and weight gain.
The question remains whether impaired SNS activity is a cause or a consequence of obesity. Blaak et al. [57] showed that isoprenaline-induced thermogenesis tended to increase after weight loss. This improvement in β-adrenoceptor-mediated thermogenesis after weight loss suggests that the impaired SNS response is a consequence of the obese state. On the other hand, Astrup et al. [58] showed that although the glucose-induced increase in energy expenditure and noradrenaline levels improved in obese subjects after 30 kg weight loss, they were still lower than in control subjects. Furthermore, Blaak et al. [57] showed that isoprenaline-induced increase in arterial NEFA concentration, as indicator for lipolysis, and muscle NEFA uptake, as indicator for lipid oxidation, remained impaired after weight reduction. This suggests that a defective SNS may rather be a primary factor leading to the development of obesity than a secondary factor resulting from the obese state.

β-adrenoceptor subtypes and obesity

Several studies have shown that the increase in energy expenditure, lipid oxidation and lipolysis are impaired in the obese during noradrenaline, adrenaline or isoprenaline infusion. Schiffelers et al. [59] found no differences between lean and obese in changes in energy expenditure during β_1-adrenergic stimulation. However during β_2-adrenergic stimulation with salbutamol, obese subjects had a reduced increase in energy expenditure, a reduced decrease in RER, suggesting a blunted increase in lipid oxidation, and a reduced increase in plasma NEFA and glycerol levels, suggesting a reduced lipolytic response. This was in line with other studies [26,31,56] which found similarly impaired responses during sympathetic activation in the obese. In *in*

vitro studies, similar results are found with respect to lipolysis. Glycerol release from subcutaneous abdominal fat cells from normal weight and overweight women was similar after incubation with dobutamine (β_1-adrenergic stimulation), but after incubation with isoprenaline (non-selective β-adrenergic stimulation) or terbutaline (β_2-adrenergic stimulation), glycerol release was reduced in fat cells from the obese. This appeared to be due to a significant reduction in cell surface density of β_2-adrenoceptors, althought mRNA levels were similar in both groups [60]. In another study, lean subjects with a low isoprenaline sensitivity, as measured by *in vitro* subcutaneous abdominal fat cell lipolysis, appeared to have a lower β_2-adrenoceptor number and mRNA level compared to lean subjects with a high isoprenaline sensitivity, whereas the β_1-adrenoceptor number and mRNA levels were similar in both groups [61]. Both studies suggest that the β_2-adrenoceptor is responsible for the reduced β-adrenoceptor-mediated increase in lipolysis.

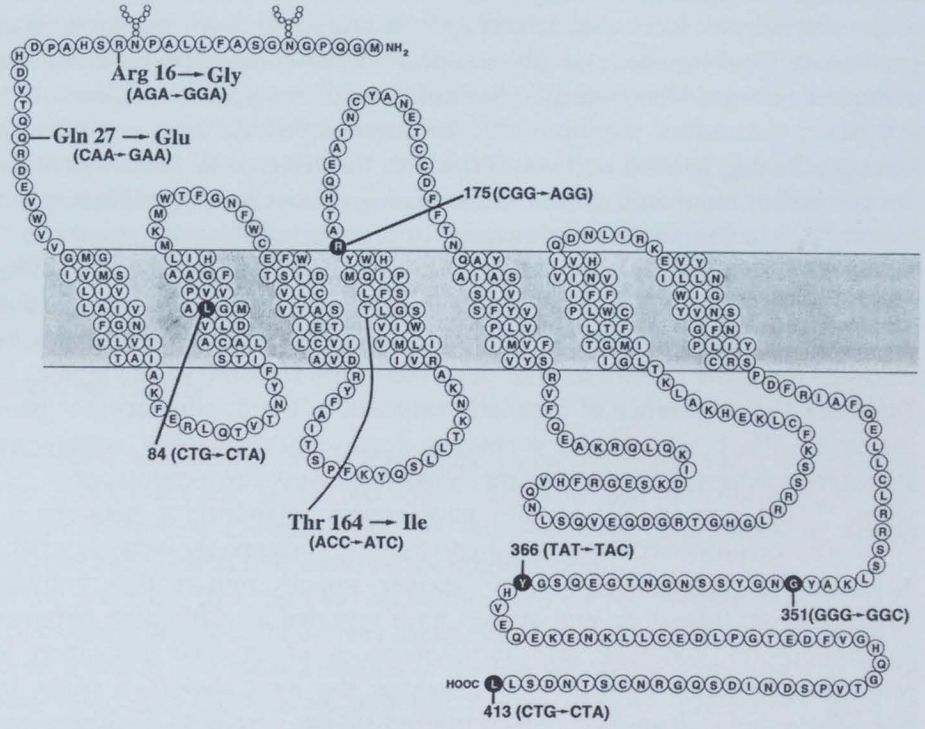

Figure 1: β_2-adrenoceptor receptor with three polymorphic codons (16, 27 and 164) adapted from Ligett [62].

β_2-adrenoceptor polymorphism functionality

Many polymorphisms have been associated with obesity or obesity-related phenotypes and the polymorphism in the β_2-adrenoceptor gene is one of them. In the coding region of the human β_2-adrenoceptor, nine polymorphisms have been identified, three of which are nonsynonymous [63]. As shown in Figure 1, the common nonsynonymous polymorphisms occur at nucleotides 47 (amino acid 16) and 79 (amino acid 27). Both display differences in allele frequencies between Caucasians and African-American [64]. Of note, the Arg 16 receptor was first cloned and has been referred to as wild-type, but is in fact the minor allelic variant.

Given their location in the amino terminus of the receptor, it is not unexpected to find no differences in agonist binding or agonist-stimulated adenylyl cyclase activities. Receptor synthesis rates and agonist-promoted internalization were not different between receptors. However, the extent of agonist-promoted down-regulation was affected by these substitutions [65]. Data suggest that the major polymorphic locus that affects agonist-promoted down-regulation is at position 16. That is, whenever Gly16 is present, down-regulation is enhanced compared to Arg16. The molecular basis of these phenotypes is not clear, but it appears to occur after the internalization process, prior to or during passage through the degradation pathway. Efforts to further explore this mechanism are somewhat hampered in that little is known about how β_2-adrenoceptors are targeted to degradation pathways.

In a study of cultured human airway smooth muscle cells natively expressing several of the β_2-adrenoceptor genotypic combinations [66], down-regulation (decrease in receptor density) promoted by 24-hour of agonist exposure followed the same pattern as that observed in the transfected cell studies [65]. Therefore the occurrence of a genetic variation in the β_2-adrenoceptor gene seems to affect the agonist promoted down-regulation after continuous stimulation, where the Arg16 variant shows the lowest down-regulation.

Some [67], but not all [68-72], studies have shown relationships between β_2-adrenoceptor polymorphisms and hypertension, presumably because of the expression of these receptors on vascular smooth muscle that regulate vasodilatation. Indeed, several studies have revealed a relationship between β_2-adrenoceptor genotype and some measure of vascular relaxation in response to agonist infusion [68,73,74]. Perhaps the most definitive study, by Boerwinkle and colleagues [71], which utilized sib-pairs from 55 pedigrees and ~2500 individuals from 589 families, revealed that the β_2-adrenoceptor polymorphisms are susceptibility loci for essential hypertension.

β_2-adrenoceptor polymorphism in human obesity

There have been several studies assessing potential associations between β_2-adrenoceptor polymorphisms and obesity [75]. These were based on the fact that β_2-adrenoceptors are expressed on white adipose tissue where activation results in lipolysis. In obese women, homozygosity for Glu27 was associated with ~20 kg higher fat mass and ~50% larger fat cells in obese compared to non-obese women [76]. However, in isolated adipocytes from such individuals, Glu27 was not associated with increased sensitivity or maximal glycerol release after terbutaline (β_2-adrenoceptor agonist) exposure *in vitro*. Instead, the sensitivity was related to the position 16 polymorphism. In another study, obesity in males was shown to be positively associated with the Gln27 polymorphism (or negatively associated with Glu27) [77]. It was concluded that gender may play a role in the influence of β_2-adrenoceptor genotype on obesity. Interestingly, the effect of position 27 polymorphisms may be modified by exercise [78] or, stated in another way, may identify patients likely to achieve weight loss with exercise [79]. Other studies however, have failed to observe any relationship between the β_2-adrenoceptor gene polymorphisms and obesity, or only a small risk for obesity [80-83]. Studies have also suggested associations with dyslipoproteinemia [83,84] and type II diabetes [83]. At this juncture, then, it is difficult to ascertain the role of β_2-adrenoceptor polymorphisms as predisposing factors for obesity. This is most likely due to the extreme clinical heterogeneity of the syndrome, gender effects, interaction with other genes, influences of other related disease such as diabetes, and environmental influences.

Outline Thesis

The research presented in this thesis focuses on the role of a polymorphism in the β_2-adrenoceptor gene (at codon 16) and the functionality of this polymorphism to contribute to the multi-factorial disturbance in energy balance and lipolysis that could be the origin of obesity.
In the first study **(chapter two)** we investigated the relationship between body composition and lipolytic activity measured *in vitro*. Also the relation between *in vitro* and *in vivo* measurements of lipolytic activity was investigated. Basal and stimulated (salbutamol and terbutaline) glycerol levels were used as measure for lipolytic activity.
Whether there was a contribution of genetic variation in the β_2-adrenoceptor gene at codon 16 to the increase in energy expenditure after β-adrenoceptor

(isoprenaline) and β_2-adrenoceptor (salbutamol) stimulation, has been described in **chapters three** and **four**.
In **chapter five** we investigated the influence of genetic variation in the β_2-adrenoceptor on diet-induced thermogenesis. We stimulated the sympathetic nervous system with a high-carbohydrate, high-protein liquid meal and measured energy expenditure over 4 hours.
We investigated the influence of overfeeding with two different diets (low-protein (3%) and normal protein (15%)) on weight gain and 24-hour energy expenditure. Uncoupling protein 3 expression and intramyocellular triacylglycerol change after overfeeding were also described in **chapter six**.
Chapter seven (general discussion) combines the different studies and puts the results in perspective.

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2

In vitro β_2-adrenoceptor-stimulated lipolysis in relation to body composition and comparison with *in vivo* stimulation

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Abstract

It has been shown that the *in vitro* plasma free fatty acid response to infusion of a β_2-adrenoceptor (β_2-AR) agonist is blunted in the obese. The aim of this study was to investigate the relation between body composition and β_2-AR stimulated activity in isolated fat cells *in vitro*. In addition we compared *in vitro* with *in vivo* β_2-AR stimulated lipolysis within subjects.

Subcutaneous abdominal fat biopsies were taken from 14 subjects (BMI range 20.7 – 33.6 kg/m^2) and these were stimulated *in vitro* with an increasing concentration of two different β_2-AR agonists, salbutamol and terbutaline. Nine subjects also participated in the second part of the study in which three increasing doses of salbutamol were infused *in vivo*. Non-esterified fatty acids (NEFA) and glycerol concentrations were measured in plasma.

No statistically significant correlation was found between body composition and β_2-AR stimulated lipolytic activity *in vitro*, although all relationships were negative. Maximal lipolytic rate (*in vitro* study) was significantly positively correlated with salbutamol-induced changes in NEFA (*in vivo* study) ($r = 0.673$, $p = 0.043$). Other correlations between *in vitro* and *in vivo* lipolytic parameters were also positive but not statistically significant, which may be related to the small sample size of the study.

To conclude, no statistically significant negative correlation between *in vitro* β_2-AR lipolytic responsiveness and increasing body mass could be demonstrated, but positive correlations between *in vitro* and *in vivo* lipolytic parameters were found. The results of the study suggest that the relationships studied are, if present, not very strong and therefore a larger number of subjects may be required to be able to draw more definitive conclusions.

Introduction

Previous work from our group has shown a blunted β-adrenoceptor-mediated stimulation of lipolysis, based on changes in plasma concentrations of non-esterified fatty acids (NEFA) and glycerol, in subjects with increased fat mass [1]. A study by Schiffelers et al. [2] showed that this was mainly due to an impaired response to β_2-adrenoceptor (β_2-AR) stimulation, while the β_1-adrenoceptor mediated response was unaffected. In agreement with these findings other studies have reported a diminished rate of appearance of NEFA or glycerol in response to adrenaline infusion in upper-body obese women [3,4] and in obese children [5], but findings are not consistent [6-8]. A blunted increase in plasma levels or rate of appearance of NEFA and glycerol might be due to the fact that there is a reduced lipolytic response to β_2-AR stimulation at the level of the fat cell. On the other hand, β_2-AR-mediated effects that influence the release of NEFA's and glycerol from the adipocyte into the circulation or the disappearance of NEFA's and glycerol from the blood independent of the lipolytic rate may also play a role. Support for a reduced response to β_2-AR stimulation at the level of the adipocyte in obese women has been reported before [9]. Futhermore, weight loss resulted in an increase in β_2-AR sensitivity in obese women [10]. We therefore further explored the relationship between body composition and β_2-AR stimulation of adipocyte lipolysis in a population of mainly males.

We also made a direct comparison of *in vitro* β_2-AR responsiveness at the level of the adipocyte and the *in vivo* response of plasma NEFA and glycerol concentration to β_2-AR stimulation. These data are needed in order to know whether *in vivo* results on changes in NEFA and glycerol are reflecting the *in vitro* lipolytic response at the level of the adipocyte. Only two studies compared *in vitro* and *in vivo* lipolysis [11,12] and conflicting results were reported.

Therefore, the first aim of this study was to investigate the relation between body composition and β_2-AR stimulated activity in isolated fat cells. The second aim was to compare *in vitro* with *in vivo* β_2-AR stimulated lipolysis in the same subject.

Subjects and Methods

Subjects

Fourteen subjects participated in this study (11 men and 3 women, age 45 y (range 40 - 55 y), BMI 26.1 kg/m^2 (range 20.7 - 33.6 kg/m^2)). Subjects did not use medication.
The study protocol was reviewed and approved by the Ethics Committee of Maastricht University and all subjects gave informed consent before participating in the tests. All subjects participated in part 1 of the study, 9 of them also in part 2.
Physical characteristics of the subjects are summarized in table 1.

Table 1: Subject characteristics for study part 1 (*in vitro* lipolysis) and part 2 (both *in vitro* and *in vivo* lipolysis)

		Part 1 (n=14)	Part 2 (n=9)
	Weight (kg)	79.4 ± 3.1	79.9 ± 4.5
	Height (cm)	174.7 ± 2.6	172.7 ± 3.9
	BMI (kg / m^2)	26.1 ± 1.0	26.8 ± 1.3
	Fat percentage (%)	28.9 ± 2.4	30.7 ± 3.7
	Fat Mass (kg)	23.3 ± 2.4	25.0 ± 3.6
	Fat Free Mass (kg)	56.1 ± 2.3	54.9 ± 3.4
In vitro	pD_2 salbutamol (-log nM)	6.18 ± 0.30	6.32 ± 0.36
	pD_2 terbutaline (-log nM)	6.04 ± 0.62	6.42 ± 1.03
	Maximal lipolytic rate salbutamol (µmol/10^7 cells/2 h)	11.3 ± 1.9	11.1 ± 2.8
	Maximal lipolytic rate terbutaline (µmol/10^7 cells/2 h)	10.7 ± 2.0	9.3 ± 2.3
In vivo	Δ NEFA (µmol/L)		360 ± 46
	Δ glycerol (µmol/L)		47.6 ± 4.1
	AUC NEFA (mmol/L * 135min)		15.43 ± 1.87
	AUC glycerol (mmol/L * 135 min)		1.91 ± 0.22

Study protocol part 1

Subjects arrived at the laboratory in the morning after an overnight fast. Body composition was determined using hydrostatic weighing with simultaneous correction for lung volume by helium dilution. Percentage body fat was

calculated from body density according to the formula of Siri [13]. Thereafter a subcutaneous fat biopsy of about 500 mg was collected from the peri-umbilical region under local anaesthesia [14].

Isolation of fat cells and determination of cell number

The adipose tissue samples were collected in saline at room temperature and fat cells were immediately isolated by collagenase treatment as described by Rodbell [15]. Briefly, the biopsy was incubated for 80 min in a shaking bath with 200 µl collagenase (100 mg/ml) in 10 ml of Krebs-Ringer phosphate buffer (10 mmol/L, pH 7.4) supplemented with 0.4 g dialysed bovine serum albumin at 37°C, thus isolating the fat cells from the stroma cells. Fat cells were washed three times with a collagenase free buffer (with 3 g/L albumin). Then they were resuspended in buffer for lipolysis experiments. Fat cell number was determined by counting 2µl suspension 5 times using a light microscope and calculating the number in the total incubation volume.

Lipolysis experiments

A diluted cell suspension (about 5000 – 15000 cells per ml) was incubated in duplicate for two hours in the presence of increasing concentrations of two different β_2-AR agonists (salbutamol (n = 14) and terbutaline (n = 10)).
All incubations were performed at 37°C in Krebs-Ringer phosphate buffer (pH = 7.4) supplemented with glucose (1 g/l) and bovine serum albumin (66 g/l). The pharmacological agents were added at the start of the incubation. The concentration range used for each drug was from 10^{-12} to 10^{-3} mol/l.

Analytical methods

Glycerol release over 2 hour into the incubation medium was determined using a semi-automated bioluminescence assay [16]. Glycerol release is a measurement of lipolytic rate since glycerol is not reutilised by human fat cells to any significant extent. These analyses were performed at the Karolinska Institute in Huddinge, Sweden.
Lipolysis rates were related to the number of incubated cells.

Drugs and chemicals

Bovine serum albumin (fraction V), Clostridium histolyticum collagenase type I, and glycerol kinase from E. Coli were obtained from Sigma (St. Louis. MO, USA). Terbutaline (Bricanyl®) was obtained from AstraZeneca BV (Zoetermeer, The

Netherlands) and salbutamol (Ventolin®) from GlaxoWellcome (Zeist, The Netherlands).

Study protocol part 2

In part 2 of the study we investigated *in vivo* stimulation of the β_2-AR. Subjects arrived at the laboratory after an overnight fast and 2 catheters (one for administration of the drugs and one for blood sampling) were placed in the antecubital vein. Indirect calorimetry measurements with a ventilated hood system were perfomed with the subject in recumbent position. The study consisted of three study periods of 45 minutes. Before the first period subjects received a priming dose of 50 µg/kg FFM atenolol (β_1-adrenoceptor antagonist) (Tenormin, Zeneca, Ridderkerk, The Netherlands) in 5 min, after which a continuous infusion of 1.2 µg/kg FFM•min atenolol was started for the remainder of the experiment, in order to block any β_1-adrenoceptor mediated effects of the β_2-AR agonist salbutamol. After 45 min atenolol infusion, subjects additionally received consecutive infusions of 50 and 100 ng/kg FFM•min salbutamol (Ventolin, GlaxoWellcome, Zeist, The Netherlands). Each infusion period lasted 45 minutes. After 30 and 45 minutes of each study period, a blood sample was taken.

Plasma analysis

Blood samples for the determination of NEFA and glycerol were preserved with sodium-EDTA. Blood samples were immediately centrifuged for 10 min at 800 *g* at 4 °C. Plasma was rapidly frozen in liquid nitrogen and stored at -70 °C until further analysis. Plasma NEFA concentration was measured with the NEFA C kit (99475409, WAKO, Neuss, Germany), plasma glycerol concentration was measured with a glycerol kit (148270, Boehringer, Mannheim, Germany) all on a Cobas Fara centrifugal analyzer (Roche Diagnostica, Basel, Switzerland).

Data analysis

In the analysis of the *in vitro* experiments the two values of the duplos for all different doses were used and a sigmoidal dose-response curve was fitted using a fixed slope (1.0). pD_2 values were calculated using Graph Pad Prism 4 for Windows. The program calculated the EC50 value, this is the value at which 50% of maximal stimulation was reached. -Log EC50 (pD_2) was used for further statistical analyses. The highest glycerol release indicates maximal

lipolytic rate and EC50 represents the sensitivity. These values were calculated for both salbutamol and terbutaline stimulation.
The change in plasma glycerol (Δglycerol) and NEFA (ΔNEFA) levels induced by the highest dose of salbutamol (100 ng/kg FFM•min) in the *in vivo* experiments was calculated by subtracting baseline concentration from the concentration at 100 ng/kg FFM•min salbutamol. ΔNEFA and Δglycerol values as well as the area under curve above baseline (AUC) for both substrates were used from the *in vivo* study as measure of the increase in lipolysis after salbutamol stimulation.
Values are given as mean ± standard error of the mean (s.e.m.). Statistical analysis was performed by one-way analysis of variance (ANOVA) and correlations were analysed using Pearson's rho correlation. A P value < 0.05 was considered as statistically significant.

Table 2: Correlations between body composition variables and *in vitro* β_2-adrenoceptor stimulated lipolysis with salbutamol. Values are Pearson's rho and P-values (n=14)

	pD_2	P-value	Maximal lipolytic rate	P-value
Body weight	-0.275	0.342	-0.335	0.242
Fat percentage	-0.184	0.529	-0.422	0.133
Fat mass	-0.240	0.408	-0.405	0.151
BMI	-0.069	0.814	-0.413	0.142

Results

Table 1 shows the characteristics for the subjects in part 1 and in part 2 of the study.
The dose response curves for *in vitro* salbutamol and terbutaline stimulation of lipolysis in subcutaneous adipocytes can be seen in figure 1. Glycerol release expressed per 10^7 fat cells was plotted against increasing agonist concentration (salbutamol or terbutaline) (Figure 1). The pD_2 for salbutamol was 6.18 ± 0.30 (-log nM) and for terbutaline 6.04 ± 0.62 (-log nM) (P = 0.91). They were well correlated (r = 0.784, P < 0.01). Maximal lipolytic rate did not differ between salbutamol and terbutaline stimulation (11.26 ± 1.96 and 10.69 ± 2.00 μmol/10^{-7} cells/2h respectively) (P = 0.179).

Relation between in vitro lipolysis and body composition

We did not find statistically significant correlations between parameters of body weight or body composition and salbutamol-induced lipolysis, maximal lipolytic rate or lipolytic sensitivity (pD_2), although all relationships were negative (Table 2).

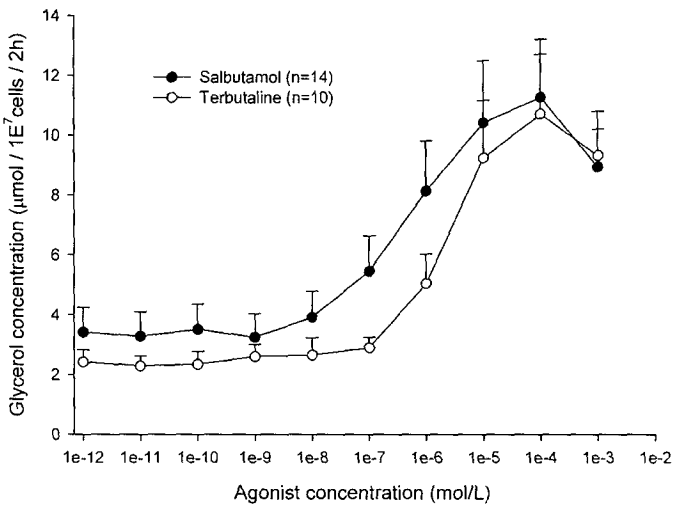

Figure 1: Glycerol release from isolated subcutaneous abdominal fat cells, corrected for fat cell number, at different concentrations of two β_2-adrenoceptor agonists (salbutamol and terbutaline)

Relation between *in vitro* and *in vivo* β_2-adrenoceptor stimulated lipolysis

Maximal lipolytic rate *in vitro* was significantly positively correlated with salbutamol-induced changes in plasma NEFA *in vivo* ($r = 0.673$, $P = 0.043$) (Table 3). The correlations of maximal lipolytic rate with the other parameters (AUC glycerol, ΔNEFA and Δglycerol) of *in vivo* lipolytic activity were also positive, although not statistically significant.

The correlations between pD_2 salbutamol ($n = 9$) and lipolytic parameters from the *in vivo* study were not statistically significant (Table 3).

Table 3: Correlations between *in vivo* salbutamol-stimulated parameters (ΔNEFA, Δglycerol, AUC NEFA, and AUC glycerol) and *in vitro* salbutamol-stimulated parameters (pD_2 and maximal lipolytic rate). Values are Pearson's rho and P-values (n=9)

In vivo \ *In vitro*	pD_2	P-value	Maximal lipolytic rate	P-value
Δ NEFA	0.462	0.211	0.543	0.131
Δ glycerol	0.640	0.087	0.419	0.301
AUC NEFA	0.379	0.215	0.673	0.047
AUC glycerol	0.353	0.391	0.241	0.565

Discussion

The aim of this study was to investigate the relation between body composition and *in vitro* β_2 adrenoceptor stimulated lipolysis and to compare β_2-AR stimulation of *in vitro* and *in vivo* lipolysis in the same subjects. Although all correlations between lipolyic parameters and body composition were negative, suggesting – if anything - lower lipolytic sensitivity and responsiveness with increasing body fatness, none of the correlations was statistically significant. The maximal lipolytic rates and lipolytic sensitivity during salbutamol and terbutaline (data not shown) stimulation were not significantly correlated with % body fat, fat mass or BMI. We investigated both salbutamol and terbutaline stimulation because of the suggested difference in selectivity for the β_2-AR. Terbutaline has been shown to be more selective for the β_2-AR (2 times higher affinity for the β_2 adrenoceptor compared to the β_1 adrenoceptor), but salbutamol has shown to have 7 times higher affinity for the β_2 adrenoceptor compared to terbutaline [17].

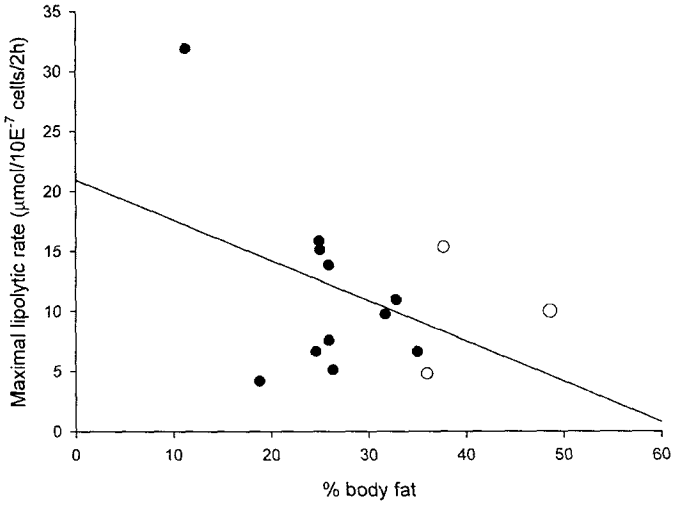

Figure 2: Correlation between maximal salbutamol stimulated lipolytic rate, corrected for number of fat cells, and body fat percentage for both males (filled circles) and females (open circles)

The absence of a statistically significant correlation between body composition and *in vitro* lipolytic response to β_2-AR stimulation may be due to the small number of subjects in this study. Reynisdottir et al. [9] showed a negative correlation between noradrenaline pD_2 and BMI in 49 female subjects. A larger number of subjects with a larger range of fat mass, BMI and % body fat than in our study may be necessary to confirm this observation. A reduced β_2 adrenergic stimulated lipolysis in obese compared to lean has been found in earlier *in vivo* studies [2,18-20]. A decreased binding capacity of the β_2-AR, as a result of reduced cell surface density of β_2-AR, in obese compared to non-obese women was associated with this decreased response in obese [9].

In the *in vitro* study the cell environment can be carefully controlled, which is often necessary when studying specific aspects of adipose tissue metabolism. However, there are also disadvantages when using *in vitro* studies. The cells are removed from their natural surroundings, resulting in absence of the effect of local tissue factors, blood flow and their effect on metabolism [21]. Therefore, we also compared the results from the *in vitro* study with the results from an *in vivo* study [22] within the same subjects. The correlations between lipolytic parameters *in vivo* (ΔNEFA, Δglycerol, AUC NEFA, and AUC glycerol) and pD_2 and maximal lipolytic rate from the *in vitro* experiments were investigated. All

parameters of *in vitro* lipolytic activity (pD_2 and maximal lipolytic rate) showed positive relations with lipolytic parameters *in vivo*, although only one correlation was statistically significant (AUC NEFA with maximal lipolytic capacity (P = 0.047)). So, we were unable to show a fully consistent relation between *in vitro* and *in vivo* lipolysis. It might be that the correlation is relatively weak as a result of local factors that influence lipolysis. Also blood flow might be an important factor because this can have a great impact on the release of NEFA and glycerol. Thus larger numbers of subjects may be necessary to get a more consistent picture.
Two other studies also describe the relation between *in vitro* and *in vivo* lipolytic parameters. Lillioja et al. [11] did not find a correlation between the *in vitro* and *in vivo* measurements. Basal free fatty acid turnover and lipid oxidation rates *in vivo* were compared with *in vitro* basal lipolytic rates of isolated abdominal fat cells. The investigators concluded that *in vitro* measurements of fat cell lipolysis cannot be used to predict *in vivo* FFA metabolism. In contrast, when *in vivo* isoprenaline-stimulated lipolysis was measured using the microdialysis technique a positive relation between *in situ* lipolysis and *in vitro* lipolysis in isolated subcutaneous adipocytes was found [12]. Results from these two studies [11,12] suggest that lipolysis measurements in isolated adipocytes predict lipolysis in fat tissue using the microdialysis technique better than whole body lipolysis measured with NEFA turnover. A possible explanation is that whole body NEFA metabolism reflects both lipolysis and oxidative and non-oxidative NEFA disposal in any part of the body.

To conclude, no statistically significant negative correlation between *in vitro* β_2-AR lipolytic responsiveness and increasing body fat could be demonstrated. The correlation between *in vitro* and *in vivo* lipolytic parameters tended to be positive. The results of the study suggest that the relationships studied are, if present, not very strong and therefore a larger number of subjects may be required to be able to draw more definitive conclusions.

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β_2-Adrenoceptor polymorphisms and salbutamol-stimulated energy expenditure

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Abstract

The β-adrenergic system is involved in the control of energy metabolism and expenditure. The β_2-adrenoceptor (β_2-AR) gene shows polymorphisms that have been associated with obesity in several studies. *In vitro* and *in vivo* studies suggest differences in β_2-AR-mediated function between these polymorphisms. The aim of this study was to investigate the influence of genetic variation in codon 16 of the β_2-AR gene on energy metabolism in humans.

Thirty-four subjects were recruited (Gly16Gly (N=13), Gly16Arg (N=16) or Arg16Arg (N=5)). The β_2-AR was stimulated with two doses salbutamol (50 and 100 ng/kg FFM•min) after blockade of the β_1-adrenoceptors with atenolol. Energy expenditure and plasma substrate and hormone concentrations were measured.

The increase in energy expenditure (ΔEE) was significantly different among groups where the Arg16Arg group showed the lowest increase ($P < 0.05$ vs Gly carriers). In a multiple regression model variations in the increase in NEFA (ΔNEFA) concentration during salbutamol infusion (partial $r = 0.51$) and the polymorphism contributed significantly to the variation in ΔEE. 35% of the variation in ΔEE was explained by these two factors.

We conclude that subjects with the Arg16Arg polymorphism of the β_2-AR gene have a reduced thermogenic response to β_2-adrenergic stimulation. Although this relatively small study needs confirmation, the findings support a role for this polymorphism in the development and maintenance of overweight and obesity.

Introduction

It is well documented that both environmental and genetic factors are involved in the onset and progression of obesity in humans. Severe obesity appears to have a particularly strong genetic component and is polygenic in nature [1]. An increasing number of polymorphisms is assumed to be associated with obesity and has been described in literature [1]. Despite intense effort, the pathways underlying these associations with obesity remain elusive. This is largely due to the complexities circumventing the process of developing obesity, which include age of onset, polygenic inheritance, genetic heterogeneity, incomplete penetrance, unknown mode of action of disease alleles, effect of ethnicity, age, gender, and the interaction with environmental factors, such as diet, physical activity or smoking status.

Of all contributing factors, the components of the sympathetic nervous system are of interest, since this system, in particular its β-adrenergic component, is involved in the control of energy metabolism and expenditure [2,3].

One of the receptor subtypes of the β-adrenergic system is the β_2-adrenoceptor (β_2-AR). It is coupled to a stimulatory G protein that promotes cAMP production, activating protein kinase A, which mediates a variety of responses, depending on the cell type. For instance, the lipolytic and glycogenolytic effects of catecholamines are mediated through members of the β_2-AR family [2,4]. One study found that the β_2-mediated thermogenic and lipolytic responses are blunted in obese compared to lean [5]. In addition, several studies [6-13] have shown an association between β_2-AR polymorphisms and weight gain, obesity or obesity-related phenotypes. Nine polymorphisms have been identified in the coding region of the human β_2-AR, three of which are nonsynonymous [14]. The two most common variants are located at codon 16 (Arg16Gly) and codon 27 (Gln27Glu) and have been studied most in relation to obesity. Both polymorphisms result in variations at the amino-terminal site of the receptor.

In vitro studies have shown differences in agonist-promoted downregulation [15], but no differences in agonist binding or agonist-stimulated adenylyl cyclase activities among these polymorphisms of the β_2-AR gene [16]. Functional consequences of these polymorphisms with respect to adipocyte lipolysis have been reported [17,18], which may play a role in obesity. So far the role of the β_2-AR polymorphisms in human energy expenditure has not been studied.

We investigated whether the polymorphism at codon 16 of the β_2-AR gene is of functional importance in human energy metabolism, by direct stimulation of this receptor with a β_2-AR agonist in an experimental setting.

Methods

Subjects

Thirty-four healthy, normal weight to moderately obese volunteers (BMI range 19.1 – 37.1 kg/m^2) participated in this study (32 men and 2 women, age 45 ± 0.8 y, BMI 27.8 ± 0.8 kg/m^2). In part (n=14) they were recruited from an existing cohort that has been described previously [13], in which the polymorphisms in the β_2-AR were known; in part they were newly recruited by advertisement (n=20). The subjects did not use medication. Physical characteristics of the subjects are summarized in table 1.

Experimental design

One week before the experimental day subjects came to the laboratory after an overnight fast and body composition was determined by hydrostatic

Table 1: Subject characteristics of groups with different polymorphisms at codon16 of the β_2-adrenoceptor gene, mean ± SEM

	Gly16Gly (n=13)	Gly16Arg (n=16)	Arg16Arg (n=5)
Gender (M/F)	12 / 1	16 / 0	4 / 1
Weight (kg)	83.6 ± 4.3	87.0 ± 3.5	86.5 ± 12.9
Height (m)	1.73 ± 0.03	1.78 ± 0.02	1.74 ± 0.05
Age (y)	46.9 ± 1.4	45.5 ± 1.3	41.5 ± 1.1
Fat percentage (%)	28.0 ± 2.6	27.4 ± 1.8	35.0 ± 1.1
Fat Mass (kg)	23.9 ± 2.7	24.6 ± 2.4	30.7 ± 5.1
Fat Free Mass (kg)	59.6 ± 3.0	62.4 ± 1.7	55.9 ± 7.8
BMI (kg/m^2)	27.8 ± 1.2	27.7 ± 1.3	28.1 ± 3.0
HOMA index	2.25 ± 0.52	2.15 ± 0.33	4.90 ± 1.84 * #

*** Significantly different between Arg16Arg and Gly16Arg ($P < 0.05$)**
 # Tendency for difference between Arg16Arg and Gly16Gly ($P = 0.06$)

weighing with simultaneous lung volume measurements (Volugraph 2000, Mijnhardt, Bunnik, The Netherlands). Body composition was calculated according to the equation of Siri [19].

Subjects were asked to refrain from strenuous exercise during 24 hours before the experiment. After an overnight fast subjects arrived at the laboratory by bus or car to minimize activity before the measurements. On arrival, a cannula was inserted into a forearm vein of each arm. One cannula was used for the infusion of drugs and the other cannula for the sampling of blood. Next, indirect calorimetry measurements were started with a ventilated hood system with the subject in recumbent position and continued for the remainder of the experiment.
The study consisted of three study periods of 45 minutes. Before the first period subjects received a priming dose of 50 µg/kg FFM atenolol (β_1-adrenoceptor antagonist) (Tenormin, Zeneca, Ridderkerk, The Netherlands) in 5 min, after which a continuous infusion of 1.2 µg/kg FFM•min atenolol was started for the remainder of the experiment. After 45 min atenolol infusion, subjects additionally received consecutive infusions of 50 and 100 ng/kg FFM•min salbutamol (Ventolin, GlaxoWellcome, Zeist, The Netherlands). Each infusion period lasted 45 minutes. After 30 and 45 minutes of each study period, a blood sample was taken.

Measurements

Whole body energy expenditure and respiratory exchange ratio (RER) were measured by indirect calorimetry, using an open-circuit ventilated hood system. In the study a home-made system was used, which is based on the analysis system for respiration chambers which has been described previously [20]. The volume of air drawn through the hood was measured by a dry-gas meter (Schlumberger, Dordrecht, The Netherlands) and the composition of the in- and outflowing air was analysed by a paramagnetic O_2 analyser (Servomex, Crowborough, UK) and an infrared CO_2 analyser (Hartmann and Braun, Frankfurt, Germany). The airflow rate and the O_2 and CO_2 concentrations of the in- and outflowing air were used to compute O_2 consumption and CO_2 production on-line through an automatic acquisition system connected to a personal computer. Energy expenditure was calculated according to the formula proposed by Weir [21]. Energy expenditure and RER values were averaged over the last 15 min of each period. The reproducibility of the measurement was within 5% as described before [22].
Total carbohydrate and fat oxidation were calculated using stoichiometric equations [23]:

Total fat oxidation (g/min) = 1.695 VO_2(L/min) – 1.701 VCO_2(L/min)
Total carbohydrate oxidation (g/min) = 4.585 VCO_2(L/min) – 3.226 VO_2(L/min)

Substrate oxidation was expressed as percentage of the contribution to total energy expenditure (1 g fat ~ 37.8 kJ and 1 g carbohydrate ~ 16.9 kJ).
Heart rate was monitored by conventional electrocardiography. Blood pressure was measured three times by an automated blood pressure device (OMRON 705CP, Hamburg, Germany) during the last 5 min of each study period. The means of these 3 measurements were used for further analysis.

Analytical methods

Genomic DNA of the subjects was extracted from leukocytes by digestion with proteinase K followed by phenol/chloroform extraction. Determination of the polymorphism was performed using a PCR-restriction fragment length polymorphism (RFLP) analysis as described before [18].
Blood samples for the determination of NEFA, glycerol, glucose, and insulin were preserved with sodium-EDTA, and those for noradrenaline and adrenaline with heparin plus glutathione (1.5% w/v). Blood samples were immediately centrifuged for 10 min at 800 *g* at 4 °C. Plasma was rapidly frozen in liquid nitrogen and stored at -70 °C until further analysis. Plasma NEFA concentration was measured with the NEFA C kit (99475409, WAKO, Neuss, Germany), plasma glycerol concentration was measured with a glycerol kit (148270, Boehringer, Mannheim, Germany), plasma glucose concentration was measured with a glucose kit (UniKit III, 07367204, Roche, Basel, Switzerland), all on a Cobas Fara centrifugal analyzer (Roche Diagnostica, Basel, Switzerland). Plasma insulin level was determined with a double antibody radio-immunoassay (Insulin RIA 100, Pharmacia, Uppsala, Sweden). The HOMA index was calculated according to Matthews et al. [24] using baseline plasma glucose and baseline plasma insulin levels. Plasma noradrenaline and adrenaline levels were determined by high performance liquid chromatography with electrochemical detection according to the method of Alberts et al. [25] using a ClinPrep kit (Recipe, Munich, Germany).

Data analysis

All data are presented as mean ± standard error of the mean (SEM). Data for energy expenditure were adjusted for FFM for group comparison [26]. The change in energy expenditure induced by the highest dose of salbutamol (100 ng/kg FFM•min) was calculated by subtracting baseline energy expenditure from the energy expenditure at 100 ng/kg FFM•min salbutamol (ΔEE). Changes in the other variables were calculated the same way.

One-way ANOVA with repeated measurements was used to analyze the effects of salbutamol administration in the total group of subjects and the three polymorphism groups. Differences among the codon 16 polymorphism groups at baseline and in response to salbutamol administration were analysed by one-way ANOVA. Post-hoc pairwise comparisons were made using Bonferroni correction.
Simple regression analysis was performed with ΔEE as the dependent variable and different parameters as independent variables (Table 3). Multiple regression analysis was conducted to estimate the independent contributions of the variables associated with ΔEE. All variables that were correlated with ΔEE in the simple regression analysis with a $P < 0.20$ were included in the analysis. In addition, the polymorphism groups were entered as dummy variables into the multiple regression model. A $P < 0.05$ was considered to be statistically significant.

Results

Responses to salbutamol infusion in the whole group

Table 2 shows changes in all parameters studied at baseline and during administration of increasing doses of salbutamol for the whole group of subjects. Energy expenditure, heart rate, and plasma noradrenaline, NEFA and glycerol concentrations showed statistically significant increases (at least $P < 0.05$). Plasma adrenaline concentrations were significantly lowered during salbutamol infusion ($P < 0.01$). No statistically significant changes in respiratory exchange ratio (RER), blood pressure (BP), and plasma glucose, lactate and insulin were found.

Baseline values and responses to salbutamol infusion in the β_2-AR codon 16 polymorphism groups

Baseline energy expenditure adjusted for FFM was similar in all polymorphism groups (4.89 ± 0.14, 4.89 ± 0.06 and 4.92 ± 0.23 kJ/min, for Gly16Gly, Gly16Arg and Arg16Arg respectively, NS). There was a significant difference in the increase in energy expenditure (ΔEE) among groups (ANOVA, $P < 0.05$) (Figure 1). Post-hoc analysis showed that the response was significantly different between the Arg16Arg and the Gly16Arg groups ($P < 0.05$) and between Arg16Arg and Gly carriers ($P < 0.05$).

Table 2: Metabolic parameters at different doses of salbutamol for the whole group (n = 34), mean ± SEM

Salbutamol (ng/kg FFM.min)	0	50	100	P-value (ANOVA)
Energy expenditure (kJ/min)	4.8 ± 0.1	5.1 ± 0.1	5.3 ± 0.1	0.037
RER	0.83 ± 0.01	0.85 ± 0.01	0.82 ± 0.01	0.183
Fat oxidation (% of total energy expenditure)	53.5 ± 2.2	48.5 ± 2.8	55.6 ± 3.2	0.179
Carbohydrate oxidation (% of total energy expenditure)	47.9 ± 2.8	53.6 ± 3.2	45.7 ± 3.5	0.181
Heart rate (beats/min)	55.9 ± 1.2	62.8 ±1.2	71.2 ± 1.4	< 0.001
Diastolic blood pressure (mmHg)	83.9 ± 1.8	81.7 ± 1.7	78.2 ± 1.7	0.075
Systolic blood pressure (mmHg)	118.1 ± 2.0	118.9 ± 1.9	121.1 ± 1.9	0.532
[Noradrenaline] (ng/L)	456.0 ± 20.6	513.3 ± 33.2	579 ± 32.7	0.014
[Adrenaline] (ng/L)	33.9 ± 3.5	24.1 ± 2.5	21.8 ± 2.1	0.006
[NEFA] (µmol/L)	366.6 ± 21.1	580.2 ± 30.8	724.9 ± 42.1	< 0.001
[Glycerol] (µmol/L)	58.5 ± 3.7	84.3 ± 5.2	106.8 ± 7.2	< 0.001
[Glucose] (mmol/L)	5.40 ± 0.12	5.36 ± 0.10	5.49 ± 0.10	0.656
[Lactate] (mmol/L)	0.97 ± 0.06	1.00 ± 0.05	1.15 ± 0.05	0.055
[Insulin] (mU/L)	10.1 ± 1.3	13.5 ± 1.8	15.2 ± 1.6	0.075

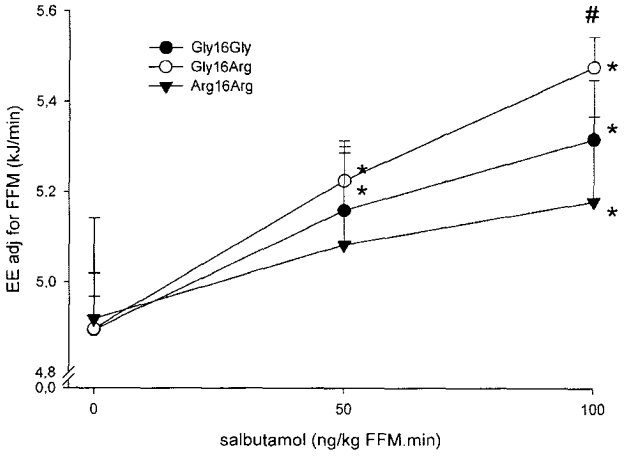

Figure 1: Energy expenditure adjusted for fat free mass for the codon 16 polymorphisms in the β_2-adrenoceptor at baseline and during infusion of salbutamol. * P < 0.05 *vs* baseline; #, P < 0.05 Arg16Arg *vs* Gly-carriers.

At baseline RER did not differ among groups, nor did ΔRER (P = 0.41) (Figure 2). Fat oxidation at baseline was not significantly different between groups (54.4 ± 3.4, 55.7 ± 3.2 and 44.6 ± 6.3 % of total energy expenditure, for Gly16Gly, Gly16Arg and Arg16Arg respectively, P = 0.25), nor was carbohydrate oxidation (47.0 ± 3.9, 45.6 ± 3.9, and 57.9 ± 7.0 % of total energy expenditure, for Gly16Gly, Gly16Arg and Arg16Arg respectively, P = 0.25). There was no difference in change of fat and carbohydrate oxidation (as percentage of total energy expenditure) in response to salbutamol among groups (ANOVA, both P = 0.43).

Plasma concentrations of NEFA, glycerol, glucose, insulin, adrenaline and noradrenaline did not differ significantly among groups at baseline nor during salbutamol infusion. The HOMA index however was significantly different between groups (ANOVA, P < 0.05), where the Arg16Arg group showed the highest HOMA index.

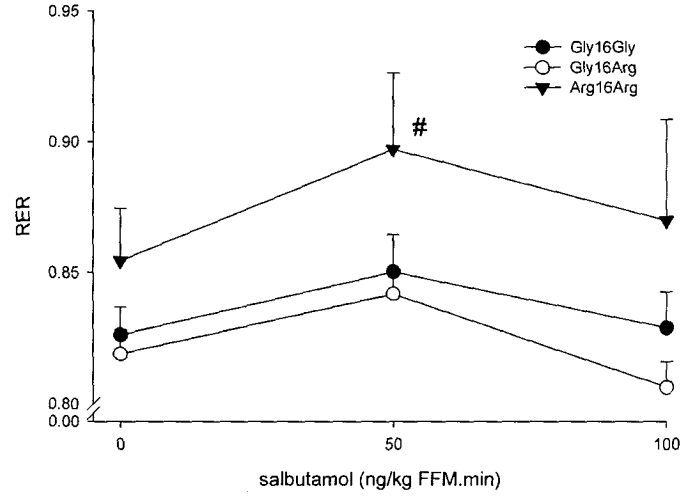

Figure 2: RER for the codon 16 polymorphisms in the β_2-adrenoceptor at baseline and during infusion of salbutamol. #, P < 0.05 Arg16Arg *vs* Gly-carriers.

During salbutamol infusion, plasma NEFA levels (Figure 3) and noradrenaline levels (Figure 4) increased significantly in all polymorphism groups ($P < 0.05$) with no significant difference among groups. Plasma concentrations of glycerol, glucose, insulin and adrenaline changed significantly in Gly16Gly and Gly16Arg groups ($P < 0.01$) but not in the Arg16Arg group; however, the responses to salbutamol infusion did not differ among groups. Plasma lactate levels were significantly higher at baseline and during salbutamol in the Arg16Arg group compared to the Gly-carriers (ANOVA, $P < 0.05$), but the lactate response to salbutamol infusion did not differ among groups (Figure 3).

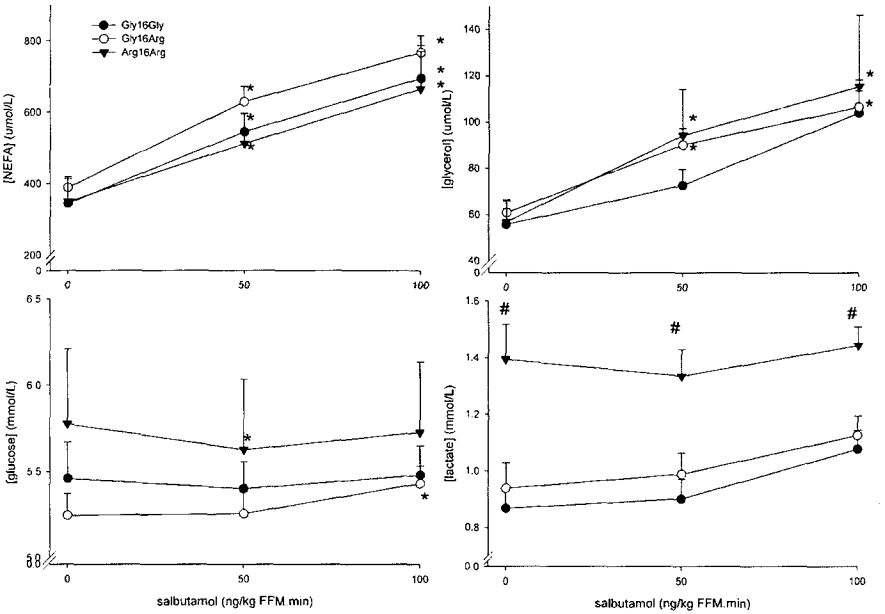

Figure 3: Plasma NEFA concentration (µmol/L), plasma glycerol concentration (µmol/L), plasma glucose concentration (mmol/L), and plasma lactate concentration (mmol/L) for the codon 16 polymorphisms in the β_2-adrenoceptor at baseline and during infusion of salbutamol. *, P < 0.05 *vs* baseline; #, P < 0.05 Arg16Arg *vs* Gly-carriers.

Baseline values for heart rate and systolic and diastolic blood pressure were not significantly different among polymorphism groups. Systolic blood pressure increased in the Gly16Gly and Arg16Arg groups (P < 0.05) but not in the Gly16Arg group (P = 0.54). Diastolic blood pressure significantly decreased in Gly16Gly and Gly16Arg group (P < 0.05) but the decrease did not reach statistical significance in the Arg16Arg group (P = 0.08). However, the changes in heart rate, systolic and diastolic blood pressure during salbutamol infusion did not differ significantly among groups (data not shown).

Table 3: Pearson correlations with delta energy expenditure (ΔEE) for the whole group (n = 34)

Variables	Correlation	P-value
Weight	-0.1	0.572
Fat percentage	-0.122	0.491
Fat mass	-0.117	0.512
Fat free mass	-0.05	0.777
BMI	-0.181	0.305
Basal [NEFA]	0.397	0.022
Basal [glycerol]	0.302	0.093
Basal [insulin]	-0.408	0.025
Δ RER	-0.392	0.022
Δ [NEFA]	0.508	0.003
Δ [glycerol]	0.435	0.013
Δ [insulin]	0.224	0.234
HOMA index	-0.425	0.019
Age	0.241	0.170

Factors contributing to the energy expenditure response to salbutamol infusion

Simple regression analysis with ΔEE as the dependent variable showed statistically significant correlations with basal NEFA and insulin concentration, ΔNEFA, Δglycerol, HOMA index and ΔRER (Table 3). Multiple stepwise regression analysis with ΔEE as the dependent variable and basal plasma NEFA and insulin concentration, ΔNEFA, HOMA index, ΔRER, age and codon 16 polymorphism as independent variables was performed subsequently. Since glycerol and NEFA are both parameters of lipolysis and are highly correlated (R-square 0.838), only basal NEFA and ΔNEFA and not basal glycerol and Δglycerol were used in the multiple regression analysis. The analysis indicated

statistically significant associations of ΔNEFA and the codon 16 polymorphism with ΔEE. No significant association with the other variables was found. The partial correlation coefficient for ΔNEFA was 0.51. The polymorphism added another 10% to the explained variance in ΔEE. The whole model therefore explained 35% of the variation in ΔEE (adjusted R-square = 0.354, $P < 0.001$). Introducing basal and Δglycerol instead of basal and ΔNEFA into the model gave similar results, ΔNEFA being replaced by Δglycerol.

Discussion

The aim of the present study was to examine the influence of polymorphisms in codon 16 of the β_2-adrenoceptor (β_2-AR) gene on energy expenditure and substrate oxidation during β_2-AR stimulation by infusing the β_2-AR agonist salbutamol. During salbutamol infusion subjects with the Arg16Arg variant of the β_2-AR gene showed a blunted increase in energy expenditure and their plasma lactate levels were higher. Fat oxidation as percentage of total energy expenditure, plasma NEFA and glycerol levels did not differ among groups, nor was there a difference in response to salbutamol. Multiple regression analysis showed that the polymorphism in codon 16 of the β_2-AR gene and plasma NEFA or glycerol change, but not RER change, % body fat or body mass index, age, HOMA index, were significantly associated with the change in energy expenditure.

The combined infusion of salbutamol and atenolol selectively stimulates the β_2-AR, as shown previously [27]. This selective stimulation resulted in the expected increases in energy expenditure, heart rate, plasma glycerol, NEFA, lactate, insulin, and noradrenaline concentrations, and in reductions in plasma adrenaline levels and diastolic blood pressure [27,28]. There was no clear change of RER during salbutamol infusion in this study. However, changes in RER have been inconsistent in previous studies [27,28].

The β_2-AR gene contains the genetic code for the receptor protein. Several variants in this gene have been described in literature [14]. Three of the single-nucleotide polymorphisms (SNPs), at nucleotides 46, 79 and 491, lead to amino-acid substitution at codons 16, 27 and 164 in the coding region of the gene and might therefore have consequences for receptor function [29]. When expressed in cells, the receptors with polymorphisms at codons 16 and 27 have been associated with differences in cellular β_2-AR trafficking [30]. The more rare polymorphism at codon 164 is associated with depressed functional coupling to Gs and reduced β_2-adrenoceptor sequestration [31]. Large et al. [18] showed that isolated abdominal subcutaneous fat cells from women homozygous for the

Arg16 polymorphism of the β_2-AR had a 5-fold lower sensitivity for β_2-AR agonist-induced lipolysis than fat cells from women heterozygous or homozygous for Gly16, independent of % body fat of the women. More recently it was shown that fat cells from subjects with different homozygous haplotypes of the β_2-AR gene differed about 250-fold in sensitivity to terbutaline-induced lipolysis [17]. The least sensitive homozygous haplotype group in this study contained the Arg variant at codon 16 [17].

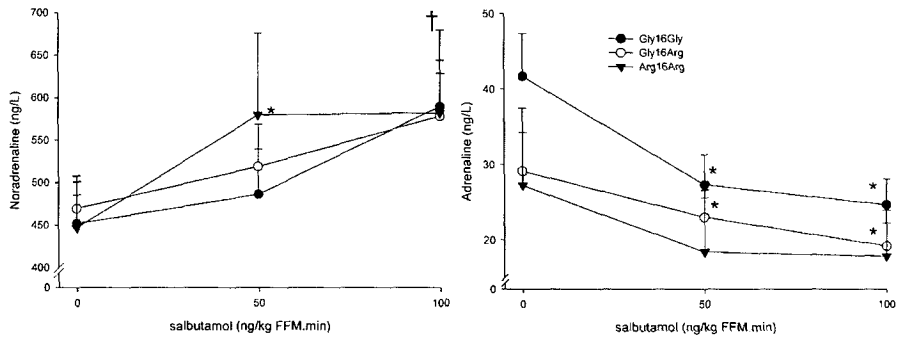

Figure 4: Plasma noradrenaline (ng/L) and adrenaline (ng/L) concentrations for the codon 16 polymorphisms in the β_2-adrenoceptor at baseline and during infusion of salbutamol. *, P < 0.05 vs baseline; † P < 0.05 vs baseline for all three groups

This study extends these findings and demonstrates a reduced β_2-AR agonist-induced thermogenic response in individuals homozygous for the Arg16 polymorphism of the β_2-AR gene. Based on changes in plasma NEFA and glycerol concentrations we could not confirm the reduced lipolytic response to β_2-AR stimulation in the Arg16Arg group, which has been reported by Large et al. [18] based on *in vitro* lipolysis. This might be related to the limitations of plasma NEFA concentration as a measure of lipolysis, since not only lipolysis, but also reesterification and NEFA oxidation affect plasma NEFA concentrations. The higher RER and plasma lactate concentrations at baseline as well as during salbutamol infusion in the Arg16Arg group suggest that their energy production relies more on carbohydrate oxidation, which would be compatible with a reduced lipolysis.

This study shows that the codon16 polymorphism has a significant effect on the salbutamol-induced thermogenic response, independent of the change in NEFA. The explanation for this effect is not directly apparent from our study. It could be that the β_2-AR-stimulated muscle and liver glycogen breakdown are

also affected by the polymorphism, thus resulting in differences in carbohydrate substrate availability and oxidation [4].

It is not surprising that the study revealed a correlation between the change in NEFA and the thermogenic response, because we and others have previously shown an increase in energy expenditure during the infusion of a triglyceride emulsion, which elevates plasma NEFA levels [28,32-34], although this is not found in all studies [35,36], which may be related to differences in patient populations and study design. In addition, part of the increase in energy expenditure induced by dobutamine, a β_1-adrenergic agonist, can be blocked by inhibiting lipolysis with nicotinic acid [37].

Previous studies have suggested that the β_2-AR is involved in the reduced β-adrenoceptor-mediated increase in lipolysis [5,38,39], lipid oxidation and thermogenesis [5] in obese compared to lean individuals. This study suggests that variations in the lipolytic response to β_2-adrenergic stimulation are associated with variations in the thermogenic response. BMI and fat percentage were negatively correlated with ΔNEFA in our study ($P<0.01$ and $P=0.06$, respectively), which supports these earlier findings. Whether the association between these variations in β_2-AR-mediated ΔNEFA and the variation in body fat are completely independent of the codon 16 polymorphism remains to be determined, but as discussed above, no association between the polymorphism and the response of plasma NEFA or glycerol was found in this study. Other factors that might have influenced the lipolytic response are age, insulin sensitivity, α_2-adrenoceptor sensitivity and other polymorphisms in the β_2-AR gene or in other genes associated with lipolysis. Aging is known to reduce β-adrenergic sensitivity [40]. The Arg16Arg group tended to be slightly, although not significantly ($P = 0.11$) younger than the other two groups. This difference in age would only tend to reduce the difference in responsiveness between the polymorphism groups. It is therefore unlikely that the differences in thermogenic sensitivity among groups are due to age differences. HOMA index as a measure for insulin sensitivity was different among polymorphism groups (Table 1) and correlated with ΔEE, but did not significantly contribute to the multiple regression model. As to be expected, the HOMA index was also associated with fat mass.

Subjects carrying the Arg16Arg polymorphism were all carriers of the Gln27Gln polymorphism of the β_2-AR gene. Of the Gly16Gly carriers 1 subject had the Gln27Gln polymorphism and 7 had the Gln27Glu polymorphism. Subjects with the Arg16Gly polymorphism were either Gln27Gln (n=7) or Gln27Glu (n=9). Limiting the analysis to the Gln27 homozygotes (n=13) revealed the same difference in thermogenic response between Gly16 carriers (n=8) and Arg16Arg carriers (n=5) as in the total group. It is therefore unlikely

that the polymorphism at codon 27 was responsible for the differences in thermogenic response between the Arg16Arg subjects and the Gly16 carriers.

We conclude that subjects with the Arg16Arg polymorphism of the β_2-AR have a reduced thermogenic response to β_2-adrenergic stimulation. Although this relatively small study needs confirmation, the findings support a role for this polymorphism in the development and maintenance of overweight and obesity, as suggested by association studies [12,17,18,41].

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4

β_2-Adrenoceptor polymorphisms and isoprenaline-stimulated energy expenditure in overweight men

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Submitted

Abstract

The β-adrenergic system is involved in the control of energy metabolism and expenditure. The β_2-adrenoceptor (β_2-AR) gene shows polymorphisms that have been associated with obesity in several studies. *In vitro* and *in vivo* studies suggest differences in β_2-AR-mediated function between these polymorphisms. The aim of this study was to investigate the influence of genetic variation in codon 16 of the β_2-AR gene on energy metabolism in humans.

Sixty-six overweight (BMI 26.2 – 48.4 kg/m^2) subjects were recruited (Gly16Gly (N=29), Gly16Arg (N=26) or Arg16Arg (N=11)). The β_2-AR was stimulated with three doses isoprenaline (0, 6 and 12 ng/kg FFM•min). Energy expenditure, plasma substrate and hormone concentrations were measured.

Energy expenditure changes were not different among polymorphism groups. None of the measured parameters contributed significantly to the increase in energy expenditure (adjusted for fat free mass). The polymorphism at codon 16 did not contribute significantly to increase in energy expenditure in the multiple regression model.

Despite previous findings, we could not show differences in increase of energy expenditure after non-selective stimulation in overweight and obese men. Therefore, we conclude that there are no differences in thermogenic response among β_2-adrenergic polymorphism groups to non-selective β-adrenergic stimulation.

Introduction

Genetic and environmental factors influence the onset and development of overweight and obesity. Perusse et al. describe a large number of polymorphisms that are associated with obesity [1]. Whether these polymorphisms have a direct effect and result in functional disturbances is unknown for most of the polymorphisms.

The sympathetic nervous system and particularly the β-adrenergic component is involved in the control of energy metabolism and expenditure [2,3]. The β_2-AR gene contains the genetic code for the β_2-receptor protein. Several variants in this gene have been described in literature [4]. Three of the single-nucleotide polymorphisms, at nucleotides 46, 79 and 491, lead to amino-acid substitution at codons 16, 27 and 164 in the coding region of the gene and might therefore have consequences for receptor function [5].

Obese subjects show a blunted increase in energy expenditure and lipolytic activity after salbutamol infusion compared to lean subjects [6]. In addition, several studies [7-11] have shown an association between β_2-AR polymorphisms and weight gain, obesity or obesity-related phenotypes. This suggests a relation between the β_2-adrenoceptor polymorphism, lipolytic activity, energy expenditure and the obesity phenotype. Recently we found that the codon 16 polymorphism in the β_2-AR explains part of the between subject variation in thermogenesis during infusion of the β_2-AR agonist, salbutamol, between individuals [12]. In that study, a blunted increase in energy expenditure was found in subjects with the Arg16Arg variant of the β_2-AR compared to Gly16-carriers. Another part of the variation was explained by differences in lipolytic response.

In contrast to salbutamol, which is selective for the β_2-AR, endogenous catecholamines stimulate all three β-adrenoceptor subtypes, although to a different extent [13]. Therefore, the aim of this study was to test whether we could replicate the previous findings, using the non-selective β-adrenergic agonist isoprenaline, in obese men carrying different polymorphisms at codon 16 of their β_2-AR gene.

Subjects and Methods

Subjects

Sixty-six overweight and obese men (BMI > 25 kg/m^2, age between 25 and 60 y) participated in this study after written informed consent was obtained. The study protocol was approved by the Ethics Committee of Maastricht University. All subjects were normotensive (diastolic blood pressure < 95 mmHg) and generally in good health. Cardiovascular and/or respiratory diseases were excluded by a medical questionnaire and physical examination. Subjects participated no more than 3 h/wk in sports activities, and none of the subjects had a physically demanding job.

Experimental design

Subjects were studied in the morning after an overnight fast. They came to the laboratory by car or by bus to minimize physical activity before the test. On arrival, a cannula was inserted into a forearm vein of each arm. One cannula was used for the infusion of isoprenaline and the other cannula for the sampling of blood. All measurements were done with the subject in recumbent position, and room temperature was kept at 21-23°C. The study protocol consisted of four study periods. After a 30-min baseline measurement, subjects received consecutive infusions of 6, 12, and 24 ng • kg fat-free mass (FFM)$^{-1}$ • min^{-1} isoprenaline (isoprenaline sulphate, Fresenius, 's Hertogenbosch, The Netherlands), each dose for 30 min. At the end of each 30-min period, a blood sample was taken. When heart rate had risen more than 30 beats/min or in case of irregularities in the ECG the infusion was stopped.

Clinical methods.

Body density was determined by hydrostatic weighing with simultaneous lung volume measurement (Volugraph 2000, Mijnhardt, Bunnik, The Netherlands), and body composition was calculated according to the equation of Siri [14].
Whole body energy expenditure and respiratory exchange ratio (RER) were measured by an open-circuit ventilated hood system (Oxycon beta, Mijnhardt, Bunnik, The Netherlands). The airflow rate and the O_2 and CO_2 concentrations of the in- and outflowing air were used to compute O_2 consumption and CO_2 production on-line through an automatic acquisition system connected to a personal computer. Energy expenditure was calculated according to the formula proposed by Weir [15]. Energy expenditure and RER values were

averaged over the last 10 min of each 30-min period during which steady state occurred.

Analytical methods

Genomic DNA of the subjects was extracted from leukocytes by digestion with proteinase K followed by phenol/chloroform extraction. Determination of the codon 16 and codon 27 polymorphism was performed using a PCR-restriction fragment length polymorphism (RFLP) analysis as described before [16].
Blood samples for the determination of nonesterified fatty acids (NEFA), glycerol, glucose, and insulin were preserved with sodium-EDTA and those for noradrenaline and adrenaline determination were preserved with heparin plus glutathione (1.5% wt/vol). Blood samples were immediately centrifuged for 10 min at 800 g at 4°C. Plasma was transferred into microtest tubes, rapidly frozen in liquid nitrogen, and stored at -70°C until further analysis. Plasma NEFA concentration was measured with the NEFA C kit (99475409, WAKO, Neuss, Germany), plasma glycerol concentration was measured with a glycerol kit (148270, Boehringer, Mannheim, Germany), and plasma glucose concentration was measured with a glucose kit (Unimate 5, 0736724, Roche Diagnostica, Basel, Switzerland), both on a Cobas-Fara centrifugal analyzer (Roche Diagnostica). Plasma insulin concentration was determined with a double antibody radioimmunoassay (Insulin RIA 100, Pharmacia, Uppsala, Sweden). Plasma noradrenaline, and adrenaline levels were determined by high-performance liquid chromatography according to the method of Alberts et al. [17]. Standard samples with known concentrations were included in each run for quality control.

Data analysis

Only baseline, 6 and 12 ng • kg FFM^{-1} • min^{-1} are analyzed, because not all subjects finished the 24 ng dosing period (see above). All data are presented as mean ± standard error of the mean (SEM). Data for energy expenditure (EE) were adjusted for FFM for group comparison [18] and are stated as adjEE. The change in adjusted energy expenditure (adjΔEE) induced by the highest dose of isoprenaline (12 ng • kg FFM^{-1} • min^{-1}) was calculated by subtracting baseline energy expenditure from the energy expenditure at 12 ng • kg FFM^{-1} • min^{-1} isoprenaline. Changes in the other variables were calculated the same way.
One-way ANOVA with repeated measurements was used to analyze the effects of isoprenaline administration in the total group of subjects and in the three

codon 16 polymorphism groups. Differences among the codon 16 polymorphism groups at baseline and in response to isoprenaline administration were analysed by one-way ANOVA. Post-hoc pairwise comparisons were made using Bonferroni correction for multiple comparison. All analysis were also performed with a codon 27 subgroup (Gln27Gln) to exclude the possible influence of this polymorphism.
Simple regression analysis was performed with adjΔEE as the dependent variable. Multiple regression analysis was conducted to estimate the independent contributions of the variables that were correlated with adjΔEE in the simple regression analysis with a $P < 0.20$. In addition, the polymorphism groups were entered as dummy variables into the multiple regression model. A $P < 0.05$ was considered to be statistically significant.

Results

Subject characteristics

Subject characteristics are described in table 1. Subjects were overweight or obese with a BMI range of 26 – 48 kg/m^2. No statistically significant differences in subject characteristics were found among polymorphism groups.

Responses to isoprenaline infusion in the whole group

Table 2 shows values of all parameters at baseline and during administration of increasing doses of isoprenaline for the whole group of subjects. Energy expenditure, plasma noradrenaline, insulin, lactate, NEFA and glycerol concentrations showed statistically significant increases (at least $P < 0.020$).
Plasma glucose, adrenaline and respiratory exchange ratio did not decrease significantly ($P > 0.20$) with isoprenaline infusion.

Table 1: Characteristics of groups with different polymorphisms at codon 16 of the β_2-adrenoceptor gene, mean ± SEM

	Gly16Gly (n=29)	Gly16Arg (n=26)	Arg16Arg (n=11)
Age (y)	42.6 ± 1.3	44.6 ± 1.7	44.0 ± 2.7
Weight (kg)	102.0 ± 3.2	102.7 ± 2.6	105.8 ± 2.9
Height (m)	1.77 ± 0.01	1.79 ± 0.01	1.81 ± 0.02
BMI (kg/m^2)	32.6 ± 0.9	32.0 ± 0.8	32.3 ± 0.68
Fat percentage (%)	32.5 ± 1.2	31.2 ± 1.0	29.2 ± 1.5
Fat Mass (kg)	33.9 ± 2.3	32.4 ± 1.7	31.3 ± 2.2
Fat Free Mass (kg)	68.2 ± 1.5	70.3 ± 1.5	74.5 ± 1.0
Waist (cm)	111.4 ± 2.9	111.4 ± 2.1	109.4 ± 1.9
Hip (cm)	106.5 ± 1.7	106.5 ± 1.1	109.4 ± 2.2
Waist / Hip ratio	1.04 ± 0.01	1.04 ± 0.02	1.00 ± 0.01
adjEnergy expenditure (kJ/min)*	6.05 ± 0.12	6.13 ± 0.12	6.06 ± 0.20
Basal RER	0.81 ± 0.01	0.81 ± 0.01	0.80 ± 0.01

*** adjusted for fat free mass**

Baseline values and responses to isoprenaline infusion in the β_2-AR codon 16 polymorphism groups

Baseline energy expenditure adjusted for FFM was similar in all polymorphism groups (6.06 ± 0.20, 6.13 ± 0.12 and 6.06 ± 0.20 kJ/min, for Gly16Gly, Gly16Arg and Arg16Arg respectively, NS). In all groups energy expenditure increased significantly ($P < 0.001$). There was no significant difference in the increase in energy expenditure (adjΔEE) among groups ($P = 0.704$) (Figure 1) and the codon 27 polymorphism did not influence this outcome.

At baseline RER did not differ among groups ($P = 0.599$). During isoprenaline infusion RER decreased significantly in the Arg16Arg group ($P = 0.023$) and the Gly16Gly group ($P = 0.041$) but not in the Gly16Arg group ($P = 0.098$). ΔRER however was not significantly different among groups ($P = 0.625$) (Figure 2).

Plasma concentrations of NEFA, glycerol, glucose, lactate, insulin, adrenaline and noradrenaline did not differ significantly among groups at baseline nor during isoprenaline infusion. The HOMA index also was not significantly different among groups (P = 0.860).
Plasma NEFA levels (Figure 3) increased significantly in all polymorphism groups (P < 0.05) and these increases (ΔNEFA) were significantly different between Gly16Arg and Gly16Gly (P = 0.008) but no significant difference between Gly16 carriers and Arg16Arg (P = 0.88) was present.

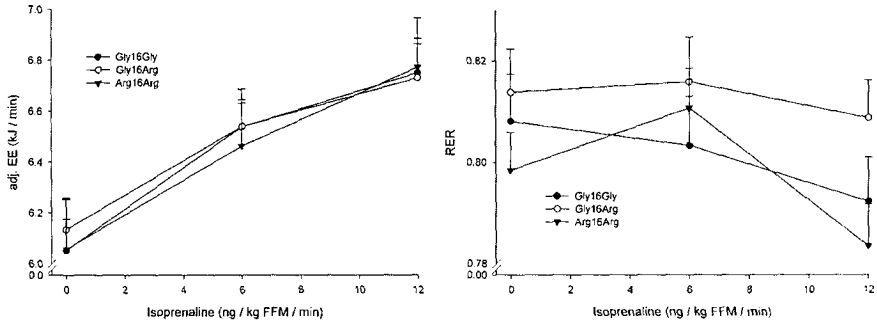

Figure 1: Energy expenditure adjusted for fat free mass and RER for the codon 16 polymorphisms in the β_2-adrenoceptor at baseline and during infusion of isoprenaline.

Plasma glycerol, insulin, glucose and noradrenaline significantly changed in all polymorphism groups during isoprenaline infusion (P < 0.027). None of these changes were statistically different among groups. Plasma adrenaline concentration only significantly changed in the Gly16Arg and Gly16Gly group (P < 0.027), but the difference among groups was not statistically significant (P = 0.749). Gly-carriers also did not differ from Arg16Arg. Plasma lactate concentration did not change significantly in any group.

Table 2: Parameters at different doses of isoprenaline for the whole group (n = 66), mean ± SEM

Isoprenaline (ng/kg FFM.min)	0	6	12	P-value (ANOVA)
Adj. Energy expenditure (kJ/min) *	6.09 ± 0.08	6.35 ± 0.08	6.59 ± 0.08	< 0.001
RER	0.81 ± 0.01	0.81 ± 0.01	0.80 ± 0.00	0.226
[Noradrenaline] (ng/L) (n=28)	386.7 ± 33.5	455.7 ± 36.0	521.6 ± 37.8	0.023
[Adrenaline] (ng/L) (n=28)	29.3 ± 2.3	23.3 ± 1.9	22.5 ± 2.5	0.229
[NEFA] (µmol/L)	477.2 ± 26.9	655.4 ± 31.5	792.7 ± 34.4	< 0.001
[Glycerol] (µmol/L)	73.7 ± 3.2	93.8 ± 4.7	104.0 ± 4.5	< 0.001
[Glucose] (mmol/L)	5.9 ± 0.2	5.8 ± 0.2	5.6 ± 0.2	0.629
[Lactate] (mmol/L)	0.97 ± 0.05	0.98 ± 0.04	1.03 ± 0.04	0.567
[Insulin] (mU/L)	12.7 ± 1.0	16.4 ± 1.4	18.1 ± 1.5	0.013

*** adjusted for fat free mass**

Factors contributing to the energy expenditure response to isoprenaline infusion

Correlation analysis with adjΔEE as the dependent variable showed no statistically significant correlations with any of the parameters (Table 3). Multiple stepwise regression analysis for the whole group with adjΔEE as the dependent variable and basal NEFA and Δglycerol concentrations as independent variables was performed subsequently. The analysis indicated no statistically significant association of any added parameter with adjΔEE. Adding the polymorphism as a dummy variable in the multiple regression model did not improve the explained variation of the model.

Table 3: Pearson correlations with the isoprenaline-induced increase in energy expenditure (adjΔEE) for the whole group (n = 66). Grey cells show variables that are added into the multiple regression analysis with P < 0.200.

Variables	Correlation	P value
Age	0.047	0.710
Weight	0.123	0.325
Fat percentage	0.022	0.860
Fat mass	0.096	0.443
Fat free mass	0.109	0.385
BMI	0.116	0.355
Basal [NEFA]	0.208	0.097
Basal [glycerol]	0.141	0.314
Basal [glucose]	0.044	0.739
Basal [insulin]	-0.006	0.970
HOMA index	0.094	0.548
Basal RER	-0.067	0.593
Δ RER	-0.006	0.963
Δ [NEFA]	0.136	0.303
Δ [glycerol]	0.242	0.102
Δ [glucose]	-0.096	0.485
Δ [insulin]	0.165	0.291

Discussion

The aim of the present study was to examine the influence of polymorphisms in codon 16 of the β_2-adrenoceptor (β_2-AR) gene on energy expenditure during β-AR stimulation by infusing the non-selective β-AR agonist isoprenaline. During isoprenaline infusion there were no significant differences between the polymorphism groups regarding the increase in energy expenditure adjusted for fat free mass (adjΔEE). No statistically significant association between adjΔEE with any of the measured parameters was found, nor could a contribution of the codon 16 polymorphism be demonstrated in a multiple regression analysis.

The infusion of isoprenaline resulted in the expected [19] increases in energy expenditure, plasma glycerol, NEFA, insulin, and noradrenaline concentrations. There was no statistically significant reduction in plasma adrenaline levels found in this study, although the changes were similar to those reported by Blaak et al. [19]. The change in RER in obese subjects was comparable between the two studies. We found, like Blaak et al. [19], that RER does not change in obese subjects after isoprenaline infusion, whereas Blaak et al. [19] describe a decrease in RER in lean subjects. Obese individuals have been shown to have a blunted capacity for fat oxidation and preferably oxidize carbohydrates [19,20]

The functionality of the polymorphisms in the β_2-AR gene has been discussed in literature before. When expressed in cells, the receptors with polymorphisms at codons 16 and 27 have been associated with differences in cellular β_2-AR trafficking [21]. Recently it was shown that isolated subcutaneous fat cells from subjects with different homozygous haplotypes of the β_2-AR gene differed about 250-fold in sensitivity to terbutaline-induced lipolysis [22]. The least sensitive homozygous haplotype group contained the Arg variant at codon 16 [22]. In a previous study [12] we found a significant contribution of the codon16 polymorphism to adjΔEE after selective stimulation of the β_2-AR with salbutamol. Arg16Arg carriers had a significantly blunted thermogenic response compared to Gly16 carriers. The absence of a difference in energy expenditure among polymorphism groups in the current study may have several reasons. One of the differences between the current and former study is that in this study only overweight and obese men (BMI range 26 – 48 kg/m^2, average BMI 32.4 ± 0.4 kg/m^2) were included compared with a broader range of BMI's (range 19 – 37 kg/m^2, average BMI 27.8 ± 0.8 kg/m^2) in the former study. The overweight state might have an effect on isoprenaline-induced changes, independent of the β_2-AR polymorphism. For instance, Blaak et al. [23] reported a significant correlation between percent body fat and the dose of isoprenaline needed to increase energy expenditure by 15 %. However, in

neither of the other two studies BMI or percent body fat was significantly associated with the thermogenic response to β-AR stimulation.
Another difference between the two studies is the type of β-AR agonist used for stimulation of energy expenditure. The increase in energy expenditure with the highest isoprenaline dose in this study (~ 20 %) was twice the salbutamol-

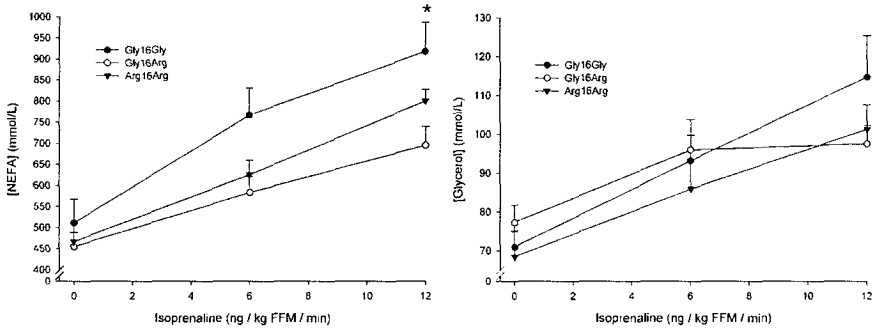

Figure 2: Plasma NEFA concentration (μmol/L) and plasma glycerol concentration (μmol/L) for the codon 16 polymorphisms in the β_2-adrenoceptor at baseline and during infusion of isoprenaline. * Significant (P < 0.05) difference in ΔNEFA between Gly16Gly and Gly16Arg and Arg-carriers.

induced increase (~ 10 %) in the other study. From a previous study [24] we know that approximately half of the isoprenaline-induced increase in energy expenditure can be blocked by a β_1-AR antagonist, suggesting that the level of β_2-AR stimulation may have been comparable in the two studies. The contribution of β_3-AR stimulation to the effect of isoprenaline is probably small [24,25]. The small range in increase in energy expenditure in the overweight subjects might have diluted the possible effect of the polymorphism.
Another possibility is that other polymorphisms in the β-AR genes have influenced the outcome of the two studies. Because isoprenaline also stimulates β_1- and β_3-AR's, it is possible that polymorphisms in these receptors have influenced the outcome of the study. For the β_1-AR gene two single nucleotide polymorphisms have been described (Ser49Gly; Gly389Arg) and for the β_3-AR gene (Trp64Arg) one that might be of functional importance. However, Leineweber et al. [26] conclude in their review that the polymorphisms in the β_1-AR gene do not appear to have functional consequences. On the other hand, the Trp64Arg polymorphism of the β_3-AR might affect functional responsiveness *in vitro* and *in vivo* and might, for this reason, contribute to an accelerated onset of metabolic disorders [26].

Two other non-synonymous polymorphisms in the β_2-AR gene (Gln27Glu and Thr164Ile) have been described in literature, from which the Gln27Glu variant has been associated with higher incidence of obesity [16,27] although not all studies can confirm this [28,29]. We checked whether this variant influenced the increase in energy expenditure in our study but could not find any contribution of this polymorphism to delta energy expenditure.

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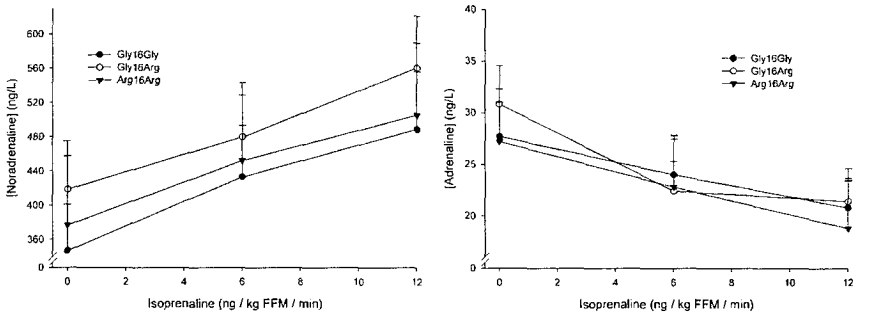

Figure 3: Plasma noradrenaline (ng/L) and adrenaline (ng/L) concentrations for the codon 16 polymorphisms in the β_2-adrenoceptor at baseline and during infusion of isoprenaline.

In our previous study [12] we found that the salbutamol-induced ΔNEFA contributed significantly to ΔEE. In the current study we found no statistically significant contribution of the variation in isoprenaline-induced ΔNEFA or Δglycerol to the variation in ΔadjEE. Although ΔNEFA was comparable between the two studies, Δglycerol was much smaller in this study, which may be suggesting a lower stimulation of lipolysis. Because in the current study only overweight subjects participated compared to subjects with wider range of BMI in previous study [12], this overweight state associated with a reduced increase in NEFA after β-AR stimulation [19], might have resulted in a blunted increase in energy expenditure.

The β_2-AR is a subclass of the β-AR family. The endogenous ligands of the β-AR are catecholamines (adrenaline and noradrenaline). Adrenaline mainly stimulates the β_2-AR. Noradrenaline is less specific for the β_2-adrenoceptor and has higher specificity for the β_1 and β_3-adrenoceptors. Isoprenaline is a non-selective β-adrenergic agonist and probably mimics endogenous β-AR stimulation under physiological conditions (e.g. exercise and ingestion of a meal) better that the selective agonist salbutamol alone.

In conclusion this study shows that none of the measured variables during β-AR stimulation contribute significantly to the thermogenic response. We could not show differences in increase of energy expenditure among β_2-AR polymorphism groups after non-selective stimulation in overweight and obese men. Therefore, we conclude that there are no differences in thermogenic response to non-selective β-adrenergic stimulation in obese men with different variants at codon 16 of the β_2-AR gene.

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5

Influence of β_2-adrenoceptor gene polymorphisms on diet-induced thermogenesis

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Abstract

The sympathetic nervous system is involved in the control of energy metabolism and expenditure. Diet-induced thermogenesis (DIT) is partly mediated by the β-adrenergic component of this system. The aim of this study was to investigate the role of genetic variation in the β_2-adrenoceptor in diet-induced thermogenesis.

24 Subjects (14 male and 10 females, BMI 26.7 ± 0.8 kg/m^2, age 45.2 ± 1.4 y) with different polymorphisms of the β_2-adrenoceptor at codon 16 (Gly16Gly, Gly16Arg or Arg16Arg) were recruited for this study. Subjects were given a high carbohydrate liquid meal and energy expenditure, respiratory exchange ratio, and plasma concentrations of non-esterified fatty acids, glycerol, glucose, insulin and catecholamines were measured before and over 4 hours after the meal.

The AUC of energy expenditure (DIT) was not significantly different among polymorphism groups, nor was the response of any of the other measured variables to the meal. In a multiple regression model the only variable that explained a significant proportion (32%) of the variation in DIT was the increase in plasma adrenaline in response to the meal ($P < 0.05$). The β_2-adrenoceptor codon 16 polymorphisms did not contribute significantly.

In conclusion, an independent contribution of the codon 16 polymorphism of the β_2-adrenoceptor gene to the variation in thermogenic response to a high carbohydrate meal could not be demonstrated. The inter-individual variation in thermogenic response to the meal was correlated with variations in the plasma adrenaline response to the meal.

Introduction

Energy expenditure is an important factor in body weight regulation. Diet-induced thermogenesis (DIT) is the energy expenditure associated with ingestion, absorption and storage of food and accounts for 10-15% to the total daily energy expenditure. DIT shows considerable inter-individual variation [1] and it can be hypothesized that a low DIT contributes to weight gain. Many studies investigated DIT in obese and lean subjects, but these studies show equivocal results [2-4]. Nevertheless, when multiple interfering factors are taken into account simultaneously, the evidence for a reduction of DIT in obesity becomes stronger [3]. In addition, several studies show no change in DIT after weight reduction, suggesting that a reduced DIT in obesity is not the consequence of the obese state per se [5,6].

In response to feeding, especially to carbohydrate (CHO) intake, sympathetic nervous system (SNS) activity increases [7,8]. The SNS-mediated thermogenic response is also referred to as facultative thermogenesis [8]. The SNS response is biphasic, with an initial increase in noradrenaline and a delayed adrenaline response [9,10]. The SNS-mediated component of DIT can be blocked by β-adrenoceptor antagonists [10,11], demonstrating the involvement of the β-adrenergic system.

β-Adrenoceptor genes are suggested to be 'candidate genes' for the development of obesity [12]. Several studies [13-20] have shown an association between polymorphisms at codons 16 and/or 27 of the β_2-adrenoceptor gene and weight gain, obesity or obesity-related phenotypes. Functional consequences of these polymorphisms with respect to adipocyte lipolysis have also been reported [21,22]. Large et al. [22] demonstrated that isolated abdominal subcutaneous fat cells from women homozygous for the Arg16 polymorphism of the β_2-adrenoceptor gene had a 5-fold lower sensitivity for the β_2-adrenoceptor agonist terbutaline-induced lipolysis than fat cells from women heterozygous or homozygous for Gly16, independent of body fat. Eriksson et al. [21] showed that homozygous haplotypes of the β_2-adrenoceptor gene differed about 250-fold in sensitivity to terbutaline-induced lipolysis, the least sensitive haplotype being homozygous for the Arg variant at codon 16. In addition, we have recently reported that the thermogenic response to infusion of the β_2-adrenoceptor agonist salbutamol was blunted in carriers of the Arg16Arg variant of the β_2-adrenoceptor gene compared to Gly16 carriers [23]. We therefore hypothesized that carriers of the Arg16Arg variant of the β_2-adrenoceptor gene might also have a reduced DIT in response to a high carbohydrate meal compared to carriers of the Gly16 variant. This might, at

least in part, explain the association between this polymorphism and obesity. This study was designed to test this hypothesis.

Subjects and Methods

Subjects

Twenty-five volunteers, 14 men and 11 women, participated in the study. They were recruited from an existing cohort in the Maastricht area in The Netherlands that has been described previously [20]. The age of the subjects ranged between 32 and 55 y. They did not use medication at the time of the study or the week before. The study protocol was reviewed and approved by the Ethics Committee of Maastricht University and all subjects gave written informed consent before participating.

Genotyping of the codon 16 polymorphism of the β_2-adrenoceptor gene

To genotype the codon 16 polymorphism of the β_2-adrenoceptor gene genomic DNA was extracted from leukocytes from each individual by digestion with proteinase K followed by phenol/chloroform extraction. Determination of the polymorphism was performed using a PCR-restriction fragment length polymorphism (RFLP) analysis as described before [22].

Experimental design

The day preceding the experimental day subjects consumed a fixed diet provided by the researchers. This diet (energy content 10.8 ± 0.3 MJ/day) consisted of 50 energy% (en%) carbohydrates, 15 en% protein and 35 en% fat, which corresponds to the average macronutrient composition of the Dutch diet. The total amount of energy each subject received was based on estimation of resting energy expenditure by the Harris-Benedict equation [24] multiplied by an estimation of the subjects' activity level. The activity level was estimated with a short questionnaire. For most subjects this activity level was set at 1.4, except when subjects reported to be at least moderately active for more than 3 hours per week, then 1.6 was used. Subjects were asked to refrain from unusual or strenuous exercise during this preceding day.
On the experimental day subjects came to the laboratory in the morning after an overnight fast. They came by car or by bus in order to limit physical activity

before the measurements. Subjects were weighed and body composition was determined by bio-impedance. Thereafter, a venous catheter was inserted in an antecubital vein for blood sampling. Then subjects were positioned under a ventilated hood in recumbent position for indirect calorimetry. The indirect calorimetry measurements continued throughout the whole experiment and were only interrupted for the consumption of the meal. Subjects were instructed to limit their movements as much as possible and not to speak during the experiment. They could watch television or a video.
After 30 minutes of rest, baseline measurements were performed: energy expenditure was measured during 30 minutes, and a baseline blood sample was collected at the end. Then subjects received an energy bolus providing 35 % of energy they consumed the preceding day (3.71 ± 0.12 MJ). This test meal consisted of two liquid formulas (total volume 593 ± 19 ml) (Meritene Polvo, Novartis Nutrition and Isostar Long Energy, Novartis Nutrition). The macronutrient composition of the test meal was 84 en% carbohydrate, 13 en% protein and 3 en% fat. A high carbohydrate and protein meal is known to stimulate sympathetic nervous system (SNS) activity [7]. The test meal was consumed within 5 minutes and measurements were continued for another 4 hours. At 10, 30, 60, 120, 180, and 240 minutes after the meal a blood sample was drawn.

Anthropometry and body composition

Weight was measured to the nearest 100 g with a digital scale (Seca Delta, Almere, The Netherlands) with clothes but without shoes. Height was known, since it had been measured before in the cohort study [20]. Total body water was measured by single frequency bioimpedance (SF-BIA) at 50 kHz, using a Xitron 400B bioimpedance analyser (Xitron Technologies Inc., San Diego, USA) [25]. Fat free mass (FFM) was calculated from weight and total body water using a prediction equation [26].

Energy expenditure, respiratory exchange ratio

Oxygen consumption (VO_2) and carbondioxide production (VCO_2) were determined using an open-circuit ventilated-hood system (Omnical, Maastricht, The Netherlands). This system is based on the analysis system for respiration chambers, which has been described previously [27]. Energy expenditure (EE) was calculated from VO_2 and VCO_2 according to the Weir-formula [28]. Respiratory Exchange Ratio (RER) is the ratio of VCO_2 and VO_2.

Analysis of blood samples

Blood samples for determination of plasma non-esterified fatty acids (NEFA), glycerol, glucose, and insulin concentrations were collected in sodium-EDTA tubes, samples for plasma noradrenaline and adrenaline concentrations in tubes containing heparin and glutathione (1.5% w/v). Blood samples were immediately centrifuged for 10 min at 800 g at 4°C. Plasma was transferred into test tubes, rapidly frozen in liquid nitrogen and stored at -80°C until further analysis.
Glucose (UniKit III, cat. no. 07367204, Roche, Basel, Switzerland) and NEFA (Wako NEFA-C test kit, Wako Chemicals, Neuss, Germany) were analysed with the COBAS FARA semi-automated analyser (Roche Diagnostica, Basel, Switzerland).
Plasma catecholamine levels (time point 0, 120 minutes and 240 minutes only) were determined by high performance liquid chromatography according to the method of Alberts et sdal. [29] using a ClinPrep kit (Recipe, Munich, Germany). Plasma insulin level was determined with a double antibody radio-immunoassay (Insulin RIA 100, Linco Research, St. Charles, USA). The HOMA index (Homeostasis model assessment), a measure of insulin sensitivity, was calculated according to Matthews et al. [30] using baseline plasma glucose and insulin levels.

Statistical methods

Data are presented as mean ± SEM. For between-group comparisons energy expenditure was adjusted for FFM. Besides absolute values of energy expenditure adjusted for FFM, the change in energy expenditure (EE) from baseline was also expressed as change relative to baseline (relativeEE = EE/baseline EE). DIT was calculated as the area under the curve of relative and absolute EE over the 4 hour post-meal period and expressed as relative DIT (relDIT) and absolute DIT (absDIT). Areas under the curve of post-meal changes from baseline were also calculated for glucose, insulin, NEFA, glycerol, noradrenaline and adrenaline concentrations. Changes over time were analysed for the whole group by repeated measures ANOVA. Analysis of variance and independent sample t-tests were used for comparisons between groups.
Simple regression analysis (Pearson's correlation) was performed with absDIT as the dependent variable and different parameters as independent variable (energy content meal, AUC for glucose, insulin, NEFA, glycerol, noradrenaline and adrenaline, baseline plasma noradrenaline and adrenaline and HOMA index) (Table 3). Multiple stepwise regression analysis, with variables of the

simple regression analysis with a P-value < 0.2 and polymorphism groups as dummy variables, was conducted to estimate the independent contributions of these variables to absDIT. A P value < 0.05 was considered to be statistically significant and all tests were performed as two-tailed tests. Statistical analyses were performed with the SPSS 11.0 statistical package.

Table 1: Subject characteristics, for the total group and the β_2-adrenoceptor polymorphism at codon 16

	All	Gly16Gly	Gly16Arg	Arg16Arg
Sex (M / F)	14 / 10	7 / 6	3 / 3	4 / 1
Estimated energy intake (MJ/day)	10.76 ± 0.30	10.79 ± 0.41	10.36 ± 0.19	11.17 ± 0.83
Energy content test meal (MJ)	3.76 ± 0.11	3.7 ± 0.14	3.62 ± 0.19	3.92 ± 0.29
Height (m)	1.75 ± 0.02	1.76 ± 0.03	1.71 ± 0.03	1.75 ± 0.05
Weight (kg)	81.7 ± 3.0	81.4 ± 4.1	80.5 ± 3.8	84.1 ± 9.8
Age (year)	45.2 ± 1.4	44.0 ± 2.1	49.5 ± 1.5	43.3 ± 2.4
BMI (kg/m^2)	26.7 ± 0.8	26.1 ± 1.1	27.7 ± 1.3	27.0 ± 2.1
Body fat (%)	28.9 ± 2.0	30.7 ± 2.7	29.6 ± 5.1	23.4 ± 2.2
Fat free mass (kg)	58.3 ± 2.9	58.3 ± 2.9	57.1 ± 5.8	64.3 ± 7.6
Resting energy expenditure (kJ/min)	4.63 ± 0.08	4.75 ± 0.11	4.55 ± 0.10	4.42 ± 0.21

No significant differences among polymorphism groups, values mean ± SEM

Results

Responses to the meal in the whole group

One subject could only consume half of the test meal and was therefore excluded from the analysis. Data on 24 subjects are reported. Their characteristics are shown in table 1. After the test meal, energy expenditure increased significantly in the whole group. After 120 minutes energy expenditure was significantly higher than baseline energy expenditure (5.61 ± 0.10 kJ/min adj. for FFM vs. 4.63 ± 0.08 kJ/min adj. for FFM respectively) ($P < 0.001$) (Figure 1). After 240 minutes energy expenditure was lower (5.34 ± 0.09 kJ/min) than at 120 minutes ($P < 0.01$), however it was still significantly higher than baseline energy expenditure ($P < 0.001$) (Figure 1). Respiratory exchange ratio (RER) increased after the meal and remained elevated over the 4-hour post-meal period ($t = 0$ vs. $t = 240$ min, $P < 0.001$) (Figure 2). Changes in plasma concentrations of metabolites and insulin are shown in figure 3. Plasma glycerol and NEFA levels were significantly lowered over the whole post-meal period ($t = 0$ vs. $t = 240$ min, $P < 0.001$). Plasma glucose concentration was significantly increased at 60 minutes compared to baseline level ($P < 0.001$) and was still higher at 240 minutes compared to baseline ($P < 0.05$). Plasma insulin levels were significantly increased at 60 minutes ($P < 0.001$). The insulin concentration increased after the meal and started to decrease 120 minutes after the meal but was still higher than baseline at 240 minutes ($P < 0.001$).
Plasma noradrenaline levels (Figure 4) were significantly elevated at 120 minutes and 240 minutes after the meal compared to baseline levels ($P < 0.05$). Plasma adrenaline levels (Figure 4) however were not significantly different from baseline at 120 minutes ($P = 0.08$). At 240 minutes, however, plasma adrenaline levels were significantly higher compared to 120 minutes ($P < 0.01$).

Table 2: Absolute DIT (absDIT) and relative DIT (relDIT) in the polymorphism groups, mean ± SEM. absDIT was calculated as AUC of energy expenditure adjusted for FFM above Resting Metabolic Rate (RMR). relDIT was calculated as AUC of relative energy expenditure (EE/RMR).

	Gly16Gly	Gly16Arg	Arg16Arg
	N = 13	N = 6	N = 5
absDIT (kJ/4 h)	175.1 ± 14.6	147.5 ± 10.8	232.2 ± 14.3
relDIT (%/4 h)	15.5 ± 1.5	13.5 ± 1.1	22.7 ± 6.4

Table 3: Pearson correlations (r) of different variables with absDIT, and corresponding P-values.

	Correlation	P-value
Energy content test meal	0.459	0.024*
AUC Glucose	0.116	0.606
AUC Insulin	0.292	0.177
AUC NEFA	0.182	0.442
AUC Glycerol	-0.142	0.528
Baseline plasma noradrenaline	0.395	0.056
Baseline plasma adrenaline	0.096	0.656
AUC noradrenaline	0.109	0.620
AUC adrenaline	0.595	0.003*
HOMA index	0.260	0.220
Fat%	-0.272	0.199
BMI	0.141	0.512

P-values < 0.2 are colored gray and * is P-values < 0.05.

Baseline values and responses to the meal in the β_2-adrenoceptor codon 16 polymorphism groups

Baseline energy expenditure was similar in all polymorphism groups (4.75 ± 0.11, 4.55 ± 0.10 and 4.42 ± 0.21 kJ/min adjusted for FFM, for Gly16Gly, Gly16Arg and Arg16Arg respectively, NS). There was no significant difference in absDIT (P = 0.125) (Figure 1) or relDIT (P = 0.131) between groups (Table 2). Additionally we compared Gly-carriers (Gly16Arg and Gly16Gly) with Arg16Arg individuals and found a trend (P = 0.06) for Arg16Arg having a higher absDIT and a statistically higher relDIT (P = 0.048) compared to Gly-carriers. None of the other parameters differed statistically significantly among the three groups, either at baseline or with respect to meal-induced changes (Figures 2 to 4).

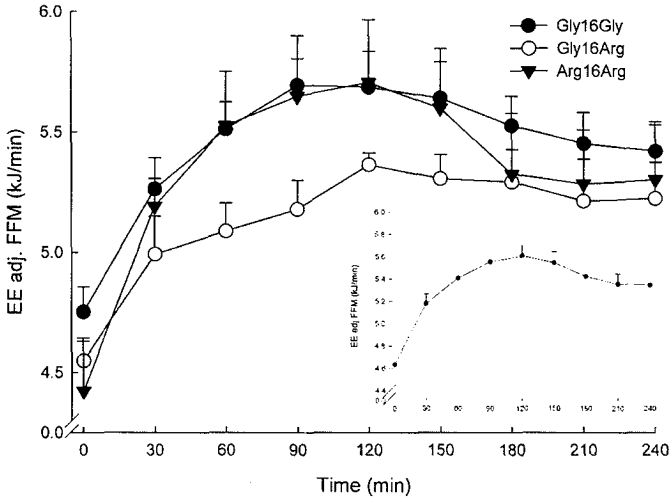

Figure 1: Energy expenditure adjusted for FFM (kJ/min.kg FFM) for the three genetic variations in the β_2-adrenoceptor, before (t=0) and after (30 minute intervals) a high carbohydrate meal. The meal was given directly after t=0. Mean + SEM.

Explanation of energy expenditure response to the meal

Of all tested variables, the change in energy expenditure after the meal (absDIT) was significantly correlated with the change in adrenaline concentration (AUC adrenaline) (P = 0.003) and with the energy content of the meal (P = 0.024) (Table 3). In a multiple regression model only AUC of plasma adrenaline level contributed significantly to absDIT (P = 0.003, adj. r-square = 0.323). When the polymorphism groups were entered in the regression model the dummy variables did not contribute significantly to absDIT (P > 0.20). Similar results were found for relDIT.

Discussion

The aim of this study was to investigate the influence of the codon16 variants of the β_2-adrenoceptor gene on the thermogenic response to a meal. We hypothesized that subjects with the Arg16Arg polymorphism of the β_2-adrenoceptor would have a lower DIT after the meal than subjects with the Arg16Gly or Gly16Gly polymorphisms. Our data do not support this hypothesis. In contrast, there was a higher relDIT and a trend for a higher absDIT in the Arg16Arg carriers compared to Gly-carriers. absDIT was most strongly associated with the adrenaline response to the meal and an additional independent contribution of the polymorphism could not be demonstrated.
Meal-induced thermogenesis is partly mediated by increased sympathoadrenal activity [7,31-33]. β-adrenoceptor blockade blunts meal-induced thermogenesis, especially between 2 and 4 hours after the meal [10], indicating that the β-adrenergic branch of the sympathoadrenal system is involved in this response during this period. In particular, the β_2-adrenoceptor seems to be important in catecholamine-induced thermogenesis [34]. The magnitude of the meal-induced stimulation of the sympathoadrenal system is mainly determined by the size of the meal and its carbohydrate and protein content [7]. Because we wanted to study differences in response to meal-induced stimulation of the β_2-adrenoceptors between subjects with different variants of the β_2-adrenoceptor gene, we tried to optimize meal-induced β-adrenergic stimulation by giving a large meal with a high carbohydrate content. Over the 4 hour post-meal period the expected increase in energy expenditure, RER, and glucose and insulin concentrations were found, accompanied by reductions in plasma NEFA and glycerol concentrations [35,36]. Differences in insulin sensitivity have been shown to affect DIT [32]. However, in our study the HOMA index, as a measure of insulin sensitivity, was not correlated with absDIT. This could be due to the fact that the variation in HOMA index in our study was relatively small. DIT has also been shown to be impaired in obese compared to lean, although results are not consistent [3]. We did not find a significant correlation between fat% and absDIT ($r = -0.272$, $P = 0.199$) nor BMI and absDIT ($r = 0.141$, $P = 0.512$), but the range in fat% and BMI in our group was relatively small.

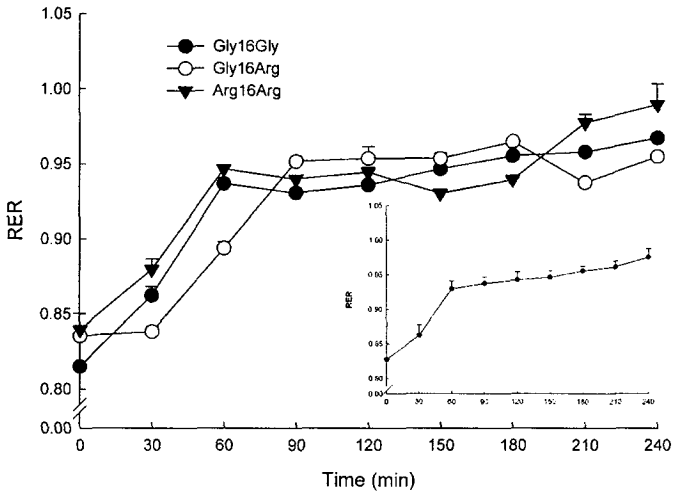

Figure 2: RER for the three genetic variations in the β_2-adrenoceptor, before (t=0) and after (30 minute intervals) a high carbohydrate meal. Mean + SEM.

Plasma noradrenaline concentration was increased at 2 and 4 hours after the meal, plasma adrenaline only at 4 hours. This early response in plasma noradrenaline levels and delayed response in plasma adrenaline levels has been shown before [35,37]. Astrup et al. [9] suggested that the delayed increase in plasma adrenaline is elicited by the decrease in plasma glucose during this period. The AUC of the post-meal changes in plasma adrenaline was the only parameter that contributed significantly to the variation in DIT (r-square = 0.32). Because adrenaline has a higher affinity for β_2-adrenoceptors than β_1- or β_3-adrenoceptors [38], the metabolic effects of adrenaline are predominantly mediated by β_2-adrenoceptors. Our data do not support a modulation of the adrenaline-induced thermogenic effect by the codon16 polymorphism of the β_2-adrenoceptor gene, as hypothesized. There was no evidence for a blunted absDIT in subjects with the Arg16Arg variant of the gene compared to Gly16 carriers. Instead, we found a trend for an increased absDIT in the Arg16Arg group compared to the Gly-carriers and relDIT was significantly higher in the Arg16Arg group. Based on the results of the multiple regression analysis this difference in DIT was partly explained by variation in adrenaline response, although we were unable to demonstrate statistically significant differences in adrenaline response between the groups.

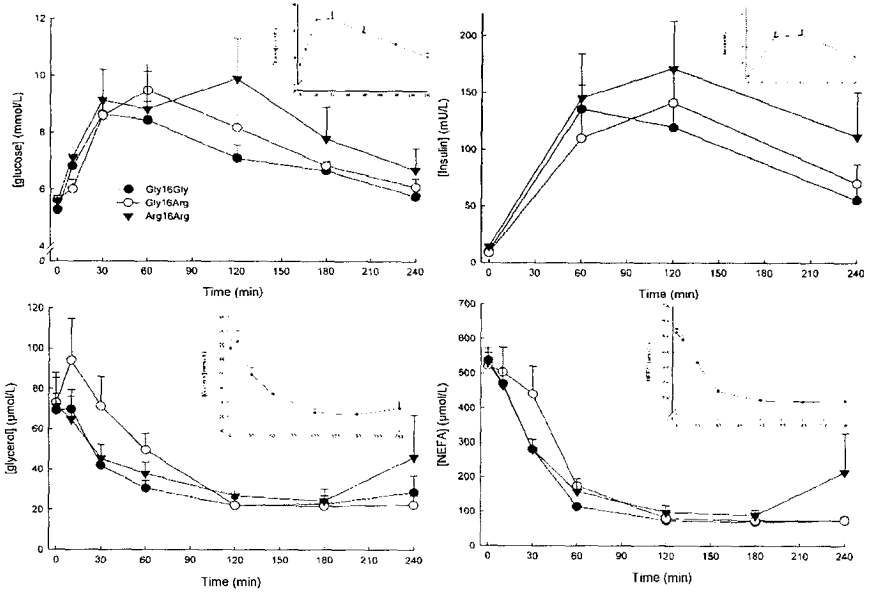

Figure 3: Plasma glucose, insulin, glycerol and NEFA levels for the three genetic variations in the β_2-adrenoceptor, before (t=0) and after (10, 30, 60, 120, 180, and 240 min) a high carbohydrate meal. Mean + SEM.

Our initial hypothesis of a reduced DIT in Arg16Arg homozygotes was based on several previous findings. The Arg16Arg polymorphism has been associated with reduced sensitivity to β_2-adrenoceptor agonist-induced lipolysis in isolated human fat cells [21,22]. Moreover, in a previous study we have shown that individuals with the Arg16Arg genotype have a blunted thermogenic response to stimulation with the β_2-adrenoceptor agonist salbutamol compared with Gly16 carriers [23]. Both factors might contribute to an increased susceptibility to weight gain and obesity in Arg16Arg carriers. The Arg16 polymorphism has indeed been associated with obesity or obesity-related phenotypes in many studies [13-20], although there are also studies that do not find this association [39,40]. The results of this study however suggest that even though the Arg16 variant of the β_2-adrenoceptor may be less sensitive to direct β_2-adrenoceptor stimulation *in vitro* and *in vivo*, the thermogenic response to a meal is increased rather than reduced in Arg16 homozygotes compared to Gly16 carriers. Whether the lower sensitivity of the Arg16Arg β_2-adrenoceptor is compensated by a higher level of sympathetic stimulation, or whether a higher level of stimulation induces a reduced responsiveness of the β_2-adrenoceptor cannot be derived from our data. The latter mechanism seems

unlikely in view of the fact that *in vitro* an enhanced agonist-promoted downregulation in the Gly16 receptor compared to the Arg16 receptor is well established [41]. However, *in vivo* the opposite has been demonstrated for isoprenaline-induced venodilatation, which was insensitive to downregulation in Gly16 homozygotes, while those homozygous for Arg16 showed significant downregulation during the 2 hour isoprenaline infusion [42]. How a reduced sensitivity of the β_2-adrenoceptor could lead to an increased activation of the sympathadrenal system is not clear from our data, but assuming that the adrenaline response is indeed linked to changes in plasma glucose [9] it is conceivable that a reduced glucose output by the liver to compensate for the fall in blood glucose late after the meal would induce a higher adrenaline response. Glucose output by the liver could be compromised by less responsive β_2-adrenoceptors involved in liver glycogenolysis or in reduced gluconeogenesis from glycerol due to compromised β_2-adrenoceptor-mediated lipolysis.

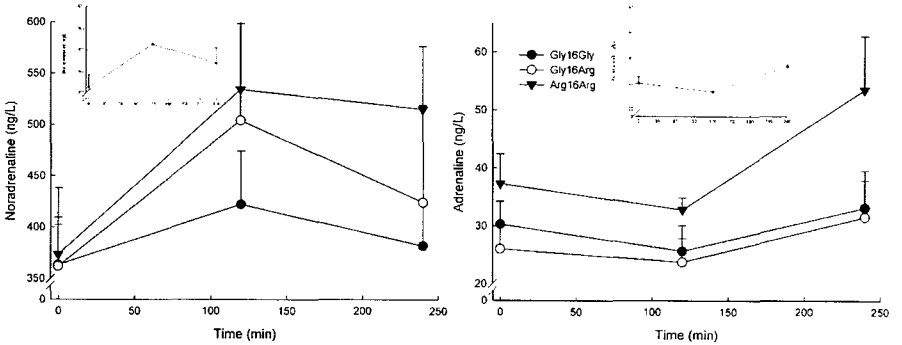

Figure 4: Plasma noradrenaline and adrenaline levels for the three genetic variations in the β_2-adrenoceptor, before (t=0) and after (120 minute intervals) a high carbohydrate meal. Mean + SEM.

In conclusion, this study shows that there is no independent contribution of the codon 16 variant of the β_2-adrenoceptor gene to the thermogenic response after a high carbohydrate meal, but that this response is positively correlated with the plasma adrenaline response to the meal.

Acknowledgment

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6

Influence of 7 days overfeeding with a low protein or normal protein diet on energy expenditure and weight gain

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Submitted

Abstract

Individual differences in weight gain as response to overfeeding have been described in several studies. The main aim of this study was to test short-term overfeeding protocols for their ability to detect metabolic susceptibility to weight gain during overfeeding in humans.

The study had a randomized cross-over design consisting of two 7-day overfeeding periods. These periods differed in diet composition: a low-protein diet (LPD) (3 energy% protein) and a normal protein diet (NPD) (15 energy% protein). Both diets overfed subjects with 1000 kcal/day above their habitual energy intake. Twenty-four-hour energy expenditure and catecholamine excretion were measured in a respiration chamber and body weight and composition were determined. Subsequently, muscle biopsies were taken for UCP3 protein measurements and NMR-spectroscopy was performed for intramyocellular triglyceride (IMTG) measurements.

Seven days of overfeeding with 1000 kcal/day on a low protein diet resulted in a body weight change of -0.17 ± 0.21 kg (n.s.), whereas a similar amount of overfeeding for 7 day on a normal-protein diet increased body weight by 0.65 ± 0.20 kg ($P < 0.05$). The difference between diets was statistically significant ($P < 0.01$). However, we did not find differences in energy expenditure between the two diets. A small, but not significant, increase in energy expenditure could not explain the fate of the extra energy intake during LPD. During NPD weight gain completely explained the fate of the excess energy intake.

In conclusion, our hypothesis that a larger compensatory increase in energy expenditure on the low-protein diet would predict a lower weight gain during the normal-protein overfeeding was not supported by our data. The developed short-term overfeeding protocol is therefore not able to test for interindividual differences in susceptibility for weight gain during overfeeding.

Introduction

Weight gain is generally due to a small but long-term positive imbalance between energy intake and energy expenditure to which several factors contribute. Hill et al. [1] proposed that behavioral susceptibility to obesity (e.g., overeating and underexercising) creates the opportunity for a positive energy balance to occur, whereas metabolic susceptibility to obesity determines the metabolic fate of excess energy when a positive balance occurs. Thus, when people are overfed and activity energy expenditure is kept the same, individual differences in metabolic susceptibility can be distinguished. Adaptive energy expenditure seems to be more influenced by energy intake than body composition [2]. These inter-individual differences in adaptive energy expenditure seem to be influenced by behavioral factors (such as Non-Exercise Activity Thermogenesis (NEAT) [3]) and genetic factors [4-6].

A number of overfeeding studies has been performed during the last century. Stock [7] analyzed several of these studies and hypothesized that the cost of weight gain is higher on unbalanced (with respect to macronutrients) overfeeding diets compared to balanced overfeeding diets. In particular a low protein diet is suggested to result in high energy cost of weight gain. The inter-individual variation in adaptive energy expenditure is relatively low using well-balanced overfeeding diets, whereas overfeeding with an unbalanced diet has been suggested to expose a range of individual responses [8]. Therefore, to investigate the difference in susceptibility to obesity in humans, different unbalanced diets can be used. We designed a protocol to compare the metabolic responses to a short-term balanced overfeeding diet and a short-term unbalanced overfeeding diet. The primary aim of this study was to make a comparison of the short-term changes in energy expenditure and weight during overfeeding with a low-protein or a normal protein diet and to test such short-term protocols for their ability to detect metabolic susceptibility to weight gain during overfeeding in humans.

Secondary, metabolic changes were studied during this overfeeding protocol. For instance, catecholamines are known to increase energy expenditure [9] and dietary catecholamine responses can influence total energy expenditure. Uncoupling proteins (UCPs) are capable of dissipating energy as heat and increasing the expression of UCP might be a strategy to lose excess energy [10]. Ukkola et al. [11] showed that genetic variation in the UCP3 gene affected body weight gain during overfeeding. We therefore tested whether UCP3 protein expression changed with overfeeding. Increased fat intake in the context of an iso-energetic diet has been shown to increase intramyocellular triglyceride

(IMTG) content in humans [12-14]. No data are available on IMTG changes during overfeeding in humans. Therefore a third objective of this study was to investigate the change in IMTG after overfeeding a diet with a normal macronutrient composition.

Subjects and Methods

Subject characteristics

Ten normal weight males (20-40 years; BMI 18.6-26.0 kg/m^2) participated in this study. All subjects were Caucasian. They were healthy and took no medication. Subjects gave their written informed consent and the Medical Ethical Committee of the Maastricht Academic Hospital approved the study. Characteristics of the subjects are summarized in table 1.

Table 1: Subject characteristics (n=10).

Age (y)	24.5 ± 1.5
Height (cm)	181.8 ± 2.0
Weight (kg)	75.9 ± 2.1
Fat %	17.1 ± 1.8
Fat mass (kg)	13.2 ± 1.6
Fat Free Mass (kg)	62.7 ± 1.60
BMI (kg/m^2)	23.0 ± 0.8
Habitual energy intake (MJ/day)	13.8 ± 0.7
Baseline energy intake (MJ/day)	11.4 ± 0.4

BMI is Body Mass Index. Mean ± SEM, n=10.

Protocol

The study had a randomized cross-over design consisting of two 7-day overfeeding periods. Prior to this, habitual energy intake was estimated and baseline 24-hour energy expenditure in a respiration chamber was determined. Subsequently, the overfeeding trial was started.

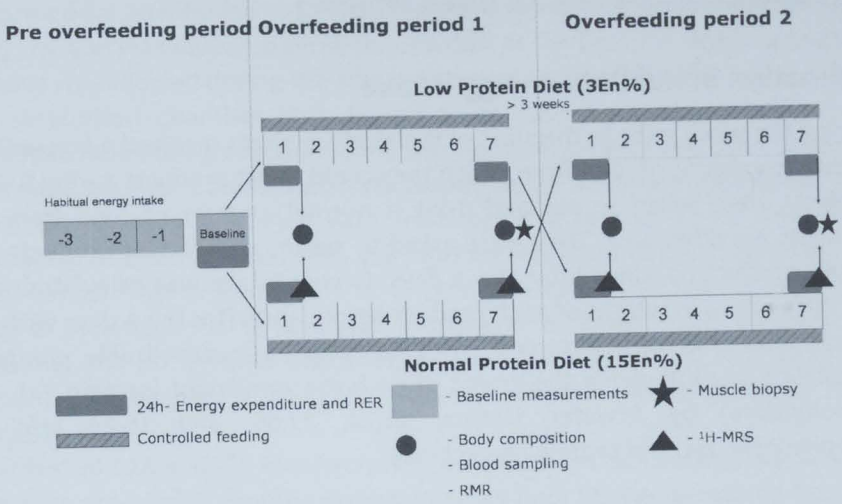

Figure 1: Protocol of the study. Low protein diet consists of 3En% protein, 67En% carbohydrate, and 30En% fat. Normal protein diet consists of 15En%, 50En% carbohydrate, and 35En% fat. RMR is resting metabolic rate. Body composition was measured by hydrostatic weighing and blood samples were drawn from an antecubital vein. Respiration chambers were used to measure 24 hour energy expenditure and substrate oxidation on day 1 and 7 of each overfeeding period. At least 3 weeks were between the two diet periods.

The subjects spent the first (day 1) and last (day 7) 24 hours of each overfeeding period in the respiration chamber. On day 2-6 subjects were in their free living environment. During the two 7 day periods subjects were overfed with 4.2 MJ/day (1000 kcal/day) above their habitual energy intake or above baseline 24-hour energy expenditure when in the respiration chamber, respectively. In random order, subjects received a low protein diet (LPD) or a normal protein diet (NPD) with at least 3 weeks between the two periods. Subjects were asked to maintain their habitual activities during the days outside the respiration chamber. Activity in the respiration chamber was standardized (see below) and all food was provided (breakfast, lunch, dinner, snacks and drinks) by the investigators. On days 2-6 subjects came to the university every day to eat breakfast and dinner and were provided with the food for the rest of the day. On the mornings of day 2 and day 8, after leavind the respiration chamber, body composition and resting metabolic rate (RMR) were measured and blood samples were taken. Additionally, on the morning of day 8 a muscle biopsy was taken and during the NPD period on day 2 and 8 intramyocellular triacylglycerol content (IMTG) was determined using ^{1}H-MRS

(magnetic resonance spectroscopy). The methods are described in detail below and a diagram of the protocol is shown in figure 1.

Estimation of habitual energy intake

During the week prior to the start of the study subjects received a box with an excessive amount of food from which they could select products during 3 days. Subjects were asked to eat and drink a normal amount of food from the products provided only. They were asked to return all left-over products and empty product packings after the 3 days. Energy intake was calculated from the differences in weight of each product before and after the 3 days with the Dutch food composition table [15]. In this table [15], metabolizable energy is calculated by multiplying the grams of each macronutrient (protein, fat, and carbohydrate) by Atwater factors; 16.74, 37.66, and 16.74 kJ/g for carbohydrate, fat, and protein respectively.

Diets

The low protein diet consisted of 3 energy% (en%) protein, 30 en% fat, and 67 en% carbohydrate and the normal protein diet consisted of 15 en% protein, 35 en% fat, and 50 en% carbohydrate. Meals and drinks were prepared for each individual subject by weighing the products with an accuracy of 1g. Drinking water was allowed ad libitum. Table 3 shows absolute amounts of macronutrient intake for the baseline and overfeeding days.

24 hour energy expenditure and substrate oxidation

To determine baseline 24 hour energy expenditure (24-hour EE), subjects stayed in the respiration chamber for 36 hours before the first overfeeding period started. The last 24 hours were used to determine baseline energy and substrate balance. Subjects were fed in energy balance with a diet composition of 50 en% carbohydrates, 35 en% fat and 15 en% protein. This is the macronutrient composition of the average Dutch diet. Cumulative energy expenditure was determined just before every meal and values were extrapolated to 24-hour values, based on previous experiments of our group [16]. In this way energy intake could be adjusted to expenditure and energy balance could be achieved over the last 24 hours of the stay in the respiration chamber.

During all stays in the respiration chamber, subjects followed the same protocol consisting of fixed times for breakfast, lunch and dinner, sedentary

activities and bench stepping exercise. The bench stepping exercise was performed for 30 minutes at a rate of 30 steps per minute with a bench height of 15 cm, and was repeated three times a day. At daytime, no sleeping or other exercise was allowed during the stay in the respiration chamber.
The respiration chamber is a 14 m^3 room furnished with a bed, chair, television, radio, telephone, intercom, wash bowl and toilet and can be used to determine 24-hour EE and substrate oxidation, as described previously [17]. In short, the room is ventilated with fresh air at a rate of 70-80 l/min. The ventilation rate is measured with a dry gas meter (Schlumberger, type G6, The Netherlands). The concentration of oxygen and carbon dioxide is measured using a paramagnetic O_2 analyzer (Hartmann & Braun, type Magnos G6, Germany) and an infrared CO_2 analyzer (Hartmann & Braun, type Uras 3G, Germany). Ingoing air is analyzed every 15 minutes and outgoing air once every 5 minutes.
For this study 24-hour EE was measured from 8.00 am to 8.00 am and 24-hour urine was collected during the same period. 24-hour EE was calculated from O_2 consumption, CO_2 production, and protein oxidation using the Weir formula [18]. Substrate oxidation was calculated using the equations of Brouwer [19]:

CHO oxidation (g/day) = $4.170 * VCO_2 - 2.965 * VO_2 - 0.390 * P$
Fat oxidation (g/day) = $1.718 * VO_2 - 1.718 * VCO_2 - 0.315 * P$
P = Protein oxidation (g/day) = $6.25 * N_{urine}$
VO_2 in L/day
VCO_2 in L/day
N_{urine} = nitrogen in urine (g/day)

Resting metabolic rate

Resting Metabolic Rate (RMR) was measured immediately after leaving the respiration chamber using an open circuit indirect calorimeter system with a ventilated hood [17]. Energy expenditure was calculated from O_2 consumption, CO_2 production and protein oxidation using the Weir formula [18]. It was assumed that protein oxidation was constant over 24 hours and therefore the 24-hour urinary nitrogen excretion (see above) was used to estimate protein oxidation during the RMR measurement.

Table 2: 24-hour energy intake and energy expenditure over time and during Low-protein diet (LPD) and normal protein diet (NPD).

Day	0	1		7	
Diet		LPD	NPD	LPD	NPD
Energy Intake (MJ/day)	11.4 ± 0.4	15.6 ± 0.4		15.6 ± 0.4	
Energy Expenditure (MJ/day)	11.0 ± 0.4	11.3 ± 0.4	11.0 ± 0.4	11.1 ± 0.4	10.9 ± 0.5

Mean ± SEM, n=10.

Body composition

Body weight was measured with a digital balance with an accuracy of 0.01 kg (Sauter, type E1200, Hamburg, Germany) after each RMR measurement. Thereafter, body density was determined by under water weighing with simultaneous assessment of lung volume with the helium dilution technique using a spirometer (Volugraph 2000, Mijnhardt, The Netherlands). Measurements were performed in triplicate and the average was used to calculate body density. Body fat percentage was calculated using the equation of Siri [20]. Fat-free mass (FFM) was calculated by subtracting fat mass from total body mass.

Intramyocellular lipid content measurement

Image-guided localized single voxel ^{1}H-MRS was performed in the vastus lateralis muscle before and immediately after the NPD overfeeding period in 6 of 10 subjects. The measurements were performed in a 1.5 T whole body scanner (Intera, Philips Medical Systems, Best, The Netherlands) with a flexible surface coil wrapped around the upper leg. To ensure that the fibers of the vastus lateralis muscle ran essentially parallel to the external magnetic field, the leg was placed at an angle of about 30° from the parallel position.

In every subject, voxels were carefully placed at the same position for all measurements. Care was taken to avoid vascular structures and adipose tissue deposits within the voxel. To reproduce the same voxel position, the longitudinal distance of the voxel from the intercondylar eminence of the knee joint was determined in a coronal image of the upper leg. The patterns of the fat distribution were used to verify the longitudinal position and as landmarks to reproduce the voxel position in the transversal plane.

^{1}H-MRS spectra from the regions of interest were acquired using a point-resolved spectroscopy sequence (PRESS) with the acquisition parameters described before [21]. The water signal was suppressed using chemically selective saturation (CHESS). The unsuppressed water signal was subsequently measured in the same voxel under the same shimming conditions and was used as a reference signal. The postprocessing has been described previously by Schrauwen-Hinderling et al. [21].

Blood samples

One sample (10 ml) was collected in EDTA-containing tubes and one (7 ml) was collected for serum. Aliquots of plasma and serum were frozen immediately in liquid nitrogen and stored at -80°C. Plasma glucose (Uni Kit III, 07367204, Roche, Basel, Switzerland), free fatty acids (FFA) (Wako NEFA-C test kit, Wako Chemicals, Neuss, Germany), glycerol (GPO-trinder 337, Sigma Diagnostics, St. Louis, USA), and triglycerides (337-40A, Sigma Diagnostics, St. Louis, USA) were analyzed with the COBAS FARA semi-automated analyzer. Serum was used to measure insulin (RIA-kit (Insulin RIA-100, Kabi-Pharmacia, Uppsala, Sweden)) and leptin (Human Leptin RIA Kit, HL-81K, St. Charles, USA).

Urinary nitrogen

24-hour urine was collected in containers with 10 ml H_2SO_4 to prevent nitrogen loss though evaporation; volume and nitrogen concentration were measured, the latter using a nitrogen analyzer (Heraeus, type CHN-O-Rapid, Hanau, Germany).

Urinary catecholamines

Samples from the 24-hour urine were used to determine 24-hour catecholamine excretion. This was done by HPLC with electrochemical detection [22].

UCP3 protein content in muscle biopsies

A percutaneous muscle biopsy was taken from the vastus lateralis muscle. After local anesthesia (0.2% xylocaine without adrenaline), a 5-mm-diameter side-cutting needle was passed through a 7-mm skin incision. The muscle biopsy was frozen immediately in liquid nitrogen and stored at -80°C until assayed.

From each muscle sample, sections approximately 40 µm times 20 µm were cut at -20°C. They were placed in 400 µl of ice-cold phosphate buffered saline (PBS) containing 0.4 mM phenyl methyl sulfonyl fluoride (PMSF) in ethanol and 1 mM EDTA pH 7.4 and vortexed for 5 s. The sample was then homogenized using a Polytron for 3 times 10 s on ice and thereafter the homogenates were sonicated for 3 times 5 s. UCP3 protein content was determined by Western blotting as previously described [23]. Cytochrome C level, as a marker of mitochondrial content, was measured comparably using a rabbit polyclonal cytochrome c antibody (BD PharMingen, Woerden, NL).

Data analysis

All results are expressed as mean ± SEM. To compare responses between and within interventions, a three-way analysis of variance (ANOVA) with diet, day and subject as factors was performed. A P-value of $P < 0.05$ was regarded as statistically significant. All analyses were performed using SPSS (release 12.0). The interaction term diet x day was used to investigate if changes depended on the type of intervention. If this interaction term did not reach statistical significance ($P < 0.05$), it was omitted from the model.

Results

Body weight and body composition

Weight changes on the different diets are shown in Figure 2. Seven days overfeeding 1000 kcal/day on a low protein diet resulted in a body weight change of -0.17 ± 0.21 kg (n.s.), whereas 7 days overfeeding 1000 kcal/day on a normal-protein diet increased body weight by 0.65 ± 0.20 kg ($P < 0.05$). The difference between diets was statistically significant ($P < 0.01$). Fat mass change during LPD was 0.09 ± 0.22 kg (n.s.), during NPD overfeeding it was 0.64 ± 0.21 kg ($P < 0.05$). There were no significant changes in fat free mass (Figure 3).

Energy expenditure and substrate oxidation

On day 0 (baseline day) subjects were fed in energy balance. Energy intake (EI) was 11.4 ± 0.4 MJ/day. The average deviation from energy balance was 0.38 ± 0.12 MJ/day (= 3.4 %).

Only during the LPD overfeeding period there were indications for an increase in 24-hour EE, but this increase was not statistically significant (Figure 4).

There was no correlation between the change in energy expenditure on the LPD and the change in body weight on the NPD (r = -0.14, and P = 0.70). Resting metabolic rate was different between the two diets on day 1 (5.51 ± 0.15 kJ/min for LPD vs. 5.13 ± 0.19 kJ/min for NPD, P = 0.05). On day 7 differences (5.32 ± 0.21 kJ/min during LPD vs. 5.27 ± 0.19 kJ/min during NPD) were not statistically significant (P = 0.49) (Figure 5). The interaction diet * day was also not statistically significant.
24-hour respiratory quotient (RQ) increased in both diet periods (baseline day 0.85 ± 0.01, day 1 LPD 0.90 ± 0.01, day 7 LPD 0.93 ± 0.02, day 1 NPD 0.88 ± 0.02, day 7 NPD 0.89 ± 0.01). The increase in RQ was more pronounced on LPD than on NPD (P < 0.001). Carbohydrate oxidation increased and fat oxidation decreased during both overfeeding periods (Table 3). Protein oxidation was decreased during LPD and increased during NPD compared to baseline values. After 7 days overfeeding on LPD the protein metabolism reached a new balance, whereas carbohydrate and fat balance remained positive on both diets (significantly different from 0, P < 0.001) (Table 3). Carbohydrate oxidation was positively correlated with carbohydrate intake (r = 0.751, P = 0.012), whereas fat oxidation was not correlated with fat intake (r = -0.154, P = 0.672).

Fasting glycerol concentrations showed a significant increase during the LPD overfeeding periods (day 1 LPD 94.3 ± 9.6 mmol/L, day 7 LPD 127.7 ± 21.2 mmol/L (P < 0.04) but there was no statistically significant difference during the NPD period (day 1 NPD 83.5 ± 9.4, and day 7 NPD 92.0 ± 11.7 P = 0.84). Fasting leptin, insulin, glucose, triglycerides and free fatty acids did not change significantly during the two diets (Table 4).

Twenty-four hour catecholamine levels

24-hour urine noradrenaline excretion was not statistically significantly different between the baseline and overfeeding days nor was it different between the first and seventh day of each overfeeding period. The same was found for adrenaline excretion. However, there was a statistically significant difference between the 24-hour noradrenaline excretion during LPD and NPD at day 7 (P = 0.047) (Table 4).

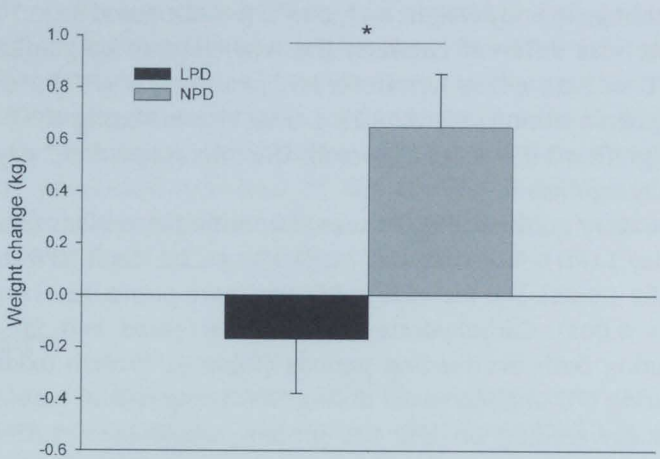

Figure 2: Weight change (in kg) after 7 days overfeeding on low protein diet (LPD, dark bar) and normal protein diet (NPD, light bar), * P < 0.01 LPD vs NPD, Mean ± SEM, n=10.

Muscle UCP3 protein content and intramyocellular lipid content

There were no statistically significant changes in UCP3 protein content corrected for mitochondrial content between baseline and day 7 of overfeeding (LPD: P = 0.91 and NPD: P = 0.32). IMTG content did not significantly change with NPD overfeeding (P = 0.49) (Table 4).

Table 3: 24-hour fat, carbohydrate, and protein intake, oxidation and balance (g/d).

		Baseline	LPD		NPD	
		0	1	7	1	7
FAT	Intake	105.8 ± 4.0	117.0 ± 3.3	117.0 ± 3.3	144.7 ± 4.0	144.7 ± 4.0
	Oxidation	120.6 ± 6.4	70.6 ± 4.2 *	54.5 ± 4.3 *#	92.9 ± 6.1 *@	80.1± 9.4
	Balance	-14.8 ± 4.3	46.4 ± 4.5 *	62.5 ± 5.8 *#	51.8 ± 4.2	64.6 ± 9.1 *
CHO	Intake	340.1 ± 13.0	619.4 ± 17.3	619.4 ± 17.3	465.0 ± 13.0	465.0 ± 13.0
	Oxidation	291.9 ± 16.9	437.0 ± 20.0 *	490.3 ± 26.9 *	350.7 ± 17.3 *@	383.4 ± 26.2 *#@
	Balance	48.2 ± 13.3	182.4 ± 15.7 *	129.1 ± 18.0	114.3 ± 12.1 *@	81.6 ± 19.4
Protein	Intake	102.0 ± 3.9	27.0 ± 0.8	27.0 ± 0.8	139.5 ± 3.9	139.5 ± 3.9
	Oxidation	80.3 ± 3.5	58.0 ± 9.1	26.3 ± 2.9 *#	86.3 ± 3.8*@	84.1 ± 5.8 #@
	Balance	21.7 ± 2.8	-31.0 ± 8.9 *	0.7 ± 2.6 *#	53.2 ± 4.0 *@	55.4 ± 10.2 *@

*** significantly different from balance day. # significantly different from day one.**
@ Significantly different from low protein diet (LPD). Mean ± SEM, n=10.

Table 4: Fasting plama or serum substrate and hormone levels (NEFA, glucose, glycerol, cholesterol, insulin and leptin) and 24-hour urinary catecholamine excretion (adrenaline and noradrenaline) after 1 and 7 days of overfeeding. IMCL change during NPD overfeeding and UCP3 protein expression at baseline and after 7 days of overfeeding.

	Baseline	LPD		NPD	
	0	1	7	1	7
Adrenaline (µg/24-hours)	11.2 ± 1.9	9.4 ± 1.4	8.3 ± 0.9	12.2 ± 1.9	12.4 ± 2.3
Noradrenaline (µg/24-hours)	43.8 ± 5.9	45.26 ± 4.6	40.0 ± 3.4	46.0 ± 3.4	53.6 ± 6.7@
IMCL (% of waterpeak)				0.35 ± 0.15	0.27± 0.16
UCP3 protein (UCP3/cytochrome C)	0.91 ± 0.11		0.90 ± 0.13		1.19 ± 0.30
Insulin (mU/L)	5,45 ± 0.72	7.48 ± 2.06	5.25 ± 0.56	6.29 ± 1.08	6.18 ± 0.97
Leptin (µg/L)	6.77 ± 1.11	4.34 ± 1.23	5.69 ± 1.15	8.39 ± 1.23	4.90 ± 0.79
Glycerol (µmol/L)	76.8 ± 11.7	91.6 ± 9.6	121.1 ± 21.2 #	79.1 ± 9.4	87.1 ± 11.6
NEFA (µmol/L)	259 ± 34	171 ± 14	224 ± 17	180 ± 14	167 ± 21
Glucose (mmol/L)	5.16 ± 0.05	5.05 ± 0.10	4.88 ± 0.06	4.94 ± 0.08	5.01 ± 0.06
Total cholesterol (mmol/L)	3.56 ± 0.22	3.50 ± 0.21	3.06 ± 0.19	3.72 ± 0.19	3.45 ± 0.15

*** significantly different from baseline day. # significantly different from day 1.**
@ significantly different from low protein diet (LPD). Mean ± SEM, n=10.

Discussion

In this study we tried to determine metabolic susceptibility during overfeeding using a short-term overfeeding protocol comparing a balanced, normal protein diet (NPD) and an unbalanced low protein diet (LPD). We hypothesized that subjects would gain less weight on the LPD than on the NPD, because the unbalanced LPD would cause a greater compensatory increase in energy expenditure [8], and that a larger compensatory increase in 24-hour EE on the LPD would predict less weight gain on the NPD. As hypothesized, weight gain during the LPD overfeeding was significantly lower than during the NPD. However, differences in compensatory increases in energy expenditure between individuals and between diets were not able to explain inter-individual differences in weight gain or differences in weight gain between diets.

Energy expenditure

During the NPD overfeeding period no increase in 24-hour EE was found. Knowing that diet-induced thermogenesis is approximately 10% of energy intake, we expected to find at least a 0.42 MJ increase in 24-hour EE due to the extra energy intake of 4.2 MJ/day during overfeeding. The only explanation for this finding is that subjects were less active in the respiration chamber during overfeeding, despite the standardized activity protocol. Only one other study also failed to show an increase in 24-hour EE after an overfeeding period [24], whereas many others [3,25-33] showed an increase in 24-hour EE. The increase in 24-hour EE varied between 0.42 MJ/day to 2.51 MJ/day (10 – 40% of excess energy intake) in these studies. Studies differed regarding amount of overfeeding, diet composition, methods for measuring energy expenditure and duration of overfeeding. The study that is most comparable to ours [26] (2 weeks overfeeding with 5.4 MJ/day), found an increase in energy expenditure of 0.75 MJ/day. The reason we did not find an increase in energy expenditure during the NPD overfeeding period might be the short overfeeding period and the relatively small excess energy intake. It might take some time before the human body adapts energy expenditure on an overfeeding diet of 4.2 MJ/day extra. Energy expenditure changes after a short-term (~7days) overfeeding period may only be reached with a high extra energy intake (+ 8 MJ/day) as in the study of Ravussin et al. [31].

During the LPD overfeeding period no significant changes in 24-hour EE were found either. The absence of an increase in energy expenditure on the LPD is hard to explain in view of the unchanged body weight and body composition of the subjects during this overfeeding regimen. Either subjects were not compliant with the diet on day 2-6, for which we have no indications, or subjects were more active on these days, for which we also don't have indications. On day 1 and 7 the intake and activity of the subjects were controlled in the respiration chamber and we can exclude large deviations from prescribed intake and activity.

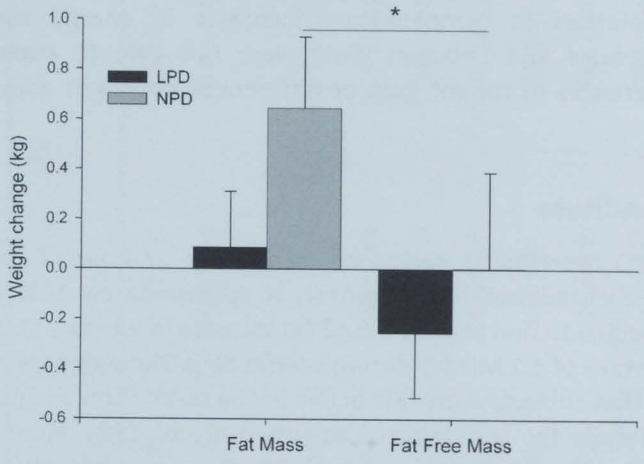

Figure 3: Change in fat mass and fat free mass after 7 days overfeeding on low protein diet (LPD, dark bar) and normal protein diet (NPD, light bar). * P = 0.09 LPD vs NPD, Mean ± SEM, n=10.

We did not measure net energy intake, since we did not correct for loss of energy in faeces. However, Webb et al. [34] and Diaz et al. [28] showed no increase in energy content of the faeces with overfeeding. Feeding a low protein diet did not influence the energy content of faeces either [35], but data regarding low protein overfeeding and energy content of the faeces are not available.
We tested a low-protein diet because Dulloo et al. [8] suggested that low-protein overfeeding is not only a potent stimulus of thermogenesis, but also an amplifier of the small inter-individual variations in thermogenesis on an affluent (normal-protein) diet. This hypothesis was based on the Gluttony experiment data [36,37] in which the energy cost of weight gain during 3-4 weeks of overfeeding a normal protein and a low protein diet was compared in five

volunteers. However, the Gluttony 1 study [36] did not measure 24-hour energy expenditure. In a controlled low protein diet intervention, Castaneda et al. [38] found no change in 24-hour EE. However, subjects were fed in energy balance in that study. As far as we know, there are no other overfeeding studies using a low-protein diet.

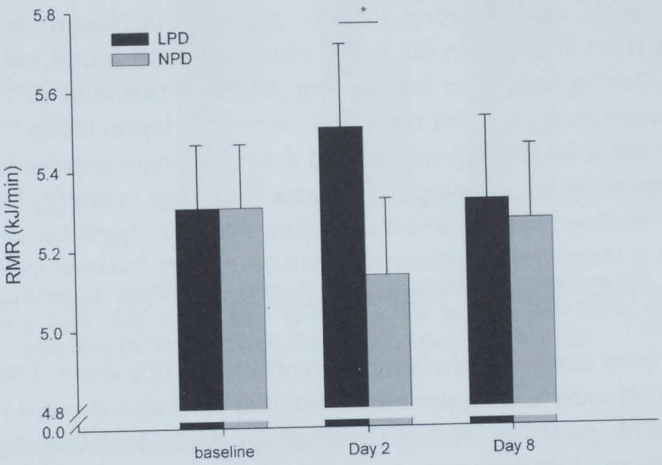

Figure 4: Energy expenditure (EE) (MJ/d) at baseline (0) and during overfeeding days (day 2 and day 8) on the low protein diet (LPD, dark bar) and normal protein diet (NPD, light bar). Mean ± SEM, n=10.

Substrate oxidation

Carbohydrate oxidation predominated during both diet periods. The RQ did not exceed 1.0 anywhere (for consecutive 3 hours) during the 24-hour period, which suggests there was no net de novo lipogenesis. A clear difference between diet periods was seen in the change in RQ during overfeeding. Fat oxidation decreased and carbohydrate oxidation increased on both diets, but this change was most apparent during LPD.

Carbohydrate oxidation showed a strong positive correlation with carbohydrate intake, where fat oxidation was reduced by overfeeding, despite increased fat intake. Jebb et al. [26] also showed the absence of a relation between fat intake and fat oxidation.

Protein oxidation decreased significantly during LPD, most probably as an adaptation to the low-protein intake. After 7 days the protein metabolism reached a new balance. In humans, these adaptations of protein oxidation to a low protein intake have been documented before in elderly women [38].

Hormonal changes

Chin-Chance et al. [39] showed an increase in leptin levels after overfeeding and a decrease with underfeeding. These changes in leptin levels were independent from changes in fat mass. Here, we were found no change in leptin levels. During long-term overfeeding (8 weeks) Levine et al. [40] showed a relation between increase in fat mass and increase in leptin levels [40]. However, in that study more fat (2.4 kg) was gained during a longer overfeeding period. So there seems to be an acute leptin response to change in energy balance and a long-term relation of leptin levels with BMI [41-43]. Van Aggel-Leijssen et al. [44] showed that a short-term moderately positive energy balance increased the amplitude of the 24-hour plasma leptin profile but not the plasma concentration.

Twenty-four hour urine-catecholamine excretion did not show differences in time during both overfeeding periods. Bandini et al. [45] also showed no change in noradrenaline excretion during overfeeding. Welle and Campbell [46] also measured urinary catecholamine excretion and it was not altered during overfeeding. We did find a significant difference in noradrenaline excretion on day 7 of overfeeding between both diet periods. This might indicate a delayed response of the SNS to the overfeeding.

UCP3 protein content and IMTG levels

We were not able to detect differences in UCP3 protein content after overfeeding, which is in line with absence of changes in 24-hour EE assuming UCP3 has uncoupling activity. It has been postulated that UCP3s main function may not be uncoupling, but limitation of the production of reactive oxygen species (ROS) or limitation of free fatty acid accumulation in the mitochondria by acting as a fatty acid anion transporter in the mitochondrial membrane, with uncoupling being a co-phenomenon of these major functions [47]. Because energy expenditure did not increase significantly during overfeeding there was probably no increased production of ROS and we also did not find increased plasma free fatty acid levels, at least after an overnight fast.

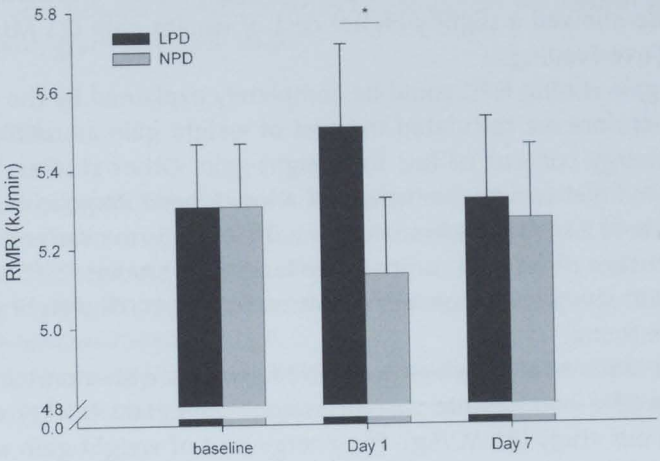

Figure 5: Resting metabolic rate (RMR) (kJ/min) at baseline (0) and during overfeeding days (day 1 and day 7) on the low protein diet (LPD, dark bar) and normal protein diet (NPD, light bar). * P = 0.05 Mean ± SEM, n=10.

During the NPD overfeeding period there was no change in IMTG content in the m. vastus lateralis. In humans no studies have been performed to investigate the influence of overfeeding on IMTG levels. Several studies which fed a high fat diet for thirty hours found increased IMTG levels compared to low-fat feeding [48,49]. However, we overfed on a diet of normal composition for 7 days. We are aware of one study that fed subjects for 7 days with a high fat diet (normal caloric intake) which increased IMTG levels [50]. Fat intake was increased from ~100 g/day on normal diet to ~210 g/day on high fat diet. In our study total fat intake increased considerably less, from 100 g/day to 143 g/day, and this might explain why we did not find an increase in IMTG levels. We are not aware of any other human overfeeding studies that have measured IMTG levels.

Weight changes

The most remarkable result of this study was the difference in weight gain between diets during the overfeeding periods. The NPD overfeeding period resulted in a significant weight gain after seven days. The cost of this weight gain (34.6 ± 36.0 MJ/kg weight gain) is comparable to that in earlier studies

[3,4,28-30,33,34,46]. Stock [7] reviewed several overfeeding studies and on average, studies with a similar amount of overfeeding and a similar diet composition as our study also showed a slightly higher cost of weight gain (41 MJ/kg weight gain) during overfeeding.
The weight gain during NPD could be completely explained by the gain in fat mass and therefore we calculated the cost of weight gain assuming a cost of 39 MJ/kg (energy content of fat) for weight-gain. Other studies [3,4,28-30,33,34,46] using the same diet composition showed a lower percentage of body weight gain as fat (50-77%). It seems that studies with short-term overfeeding show a higher percentage of fat gain compared to long-term studies [3,27-29,34]. The short duration of our study could be an explanation for the 100% weight gain as fat mass that we found.
The study by Miller et al. [36], who also overfed subjects with a protein intake of 3 en%, showed a comparable cost of weight gain (100 MJ/kg) on LPD as measured in our study (85 MJ/kg). The energy cost of weight gain was higher on LPD than on NPD and this is in agreement with Dulloo and Jacquet's hypothesis [8].
Weight gain differed significantly between the NPD and the LPD period. This difference in weight gain resulted in differences in cost of weight gain between the diets. On the LPD overfeeding period a large variation in cost of weight gain between subjects (-50-400 MJ/kg weight gain) was found. The cost of weight gain during the NPD overfeeding period showed less variation between subjects (25-50 MJ/kg weight gain). However, we failed to find a correlation between the costs of weight gain on both diets (R-square = 0.47, P = 0.81) as suggested by Dulloo and Jacquet [8]. A recent study of Joosen et al. [51] also could not find that the metabolic efficiency of weight gain was related to adaptive changes in energy expenditure.

In conclusion, 7 days overfeeding with a balanced, normal protein diet resulted in more weight gain than isocaloric overfeeding with an unbalanced, low protein diet. No compensatory increase in 24-hour energy expenditure in the respiration chamber was found in any of the overfeeding periods. Thus, our hypothesis that a larger compensatory increase in energy expenditure on the low-protein diet would predict a lower weight gain during the normal-protein overfeeding was not supported by our data. The developed short-term overfeeding protocol is therefore not able to test for interindividual differences in susceptibility for weight gain during overfeeding.

Acknowledgements

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7

General discussion

The classical overfeeding study in identical twins by Bouchard et al. [1] clearly showed a threefold difference in the ability to lay down excess calories in weight between twin pairs but hardly a difference within twin pairs. This suggests a strong genetic influence on energy efficiency in humans although multiple factors seem to contribute. The main emphasis of this thesis was on the role of genetic variability in the susceptibility for obesity. In general obesity is the result of an imbalance between energy intake and energy expenditure. We focused particularly on the energy expenditure side and genes that might be involved in the regulation of energy expenditure. The gene we especially looked at was the β_2 adrenoceptor (β_2-AR) gene and mainly the codon 16 variant (Gly16Arg) in this gene.

This gene and this particular codon variant were identified in several association studies to be associated with obesity or obesity-related phenotypes [2-9]. Besides these association studies also cell studies show that this polymorphism might result in functional differences [10,11]. Together with earlier findings that obese show blunted β2-AR stimulated increase in energy expenditure we wanted to investigate whether the β_2-AR polymorphism might be the cause of this impaired increase in energy expenditure and thus on the long term a contributing factor in the development of obesity. We performed several studies to investigate to what extent this genetic variation could contribute to an overweight phenotype. First we compared the *in vitro* β_2-AR stimulated glycerol release with *in vivo* increase in plasma glycerol and nonesterified fatty acids (NEFA's) levels during β_2-AR agonist infusion. Originally we also wanted to compare the impact of β_2-AR stimulated lipolysis in subcutaneous fat cells between the Arg16Arg with the Gly16Gly variant *in vitro* but because of the limited number of subjects participating, we were unable to perform statistical analysis with the data. In the second and third study we investigated the impact of the genetic variants of the β_2-AR gene and other influencing factors on energy expenditure *in vivo* after stimulating the β_2-AR selectively (salbutamol) and non-selectively (isoprenaline). In a more physiological situation we examined stimulation of the sympathetic nervous system by a high carbohydrate meal and the influence of the β_2-AR polymorphism on the thermogenic response to this meal. Finally we looked at the effect of two short-term overfeeding protocols (normal and low-protein diet) on energy expenditure and substrate utilisation in an attempt to develop a functional test to detect individual susceptibility to weight gain due to differences in energy efficiency.

Association studies

Our main reason to focus on the β_2-AR polymorphism, in the light of individual variation in energy expenditure was based on association studies. Many papers describe an association of the Arg16Arg variant with obesity or obesity related phenotypes [2-9,12], although not all studies can confirm this finding [6,13]. Studies vary with respect to ethnicity of the subjects, gender, and measured endpoint.

In literature the variance in codon 16 in the β_2-AR is not only associated with obesity and related phenotypes but also with cardiovascular reactivity [14,15], blood pressure [12,16-20], asthma [21,22], and even with parasitic infection [23] and pregnancy outcome [24]. But again, not all studies show an association of the polymorphism with blood pressure [25] or with asthmatic phenotype [26-28].

It is not surprising that associations have been found between β_2-AR gene polymorphisms and asthma and blood pressure because the β_2-AR is an important receptor in the lung and cardiovascular tissue. In the lung this receptor regulates bronchodilatation and in the cardiovascular system it regulates peripheral resistance by vasodilatation. These associations with other phenotypes support the suggestion that this codon 16 variance has a relevant physiological function.

However, like all case-control studies, also genetic case-control association studies suffer from a number of methodological problems like many sources of bias, unsatisfactory designation of cases and controls, which can result in false positive associations. High throughput genotyping, inadequate matching of cases and controls and multiple testing increase the risk of finding false positive associations. Confirmation of such findings by other studies has to be obtained before associations can be considered as true. In particular in the first period of genotyping there was the problem of publication bias for positive results. In addition, using Single Nucleotide Polymorphisms (SNPs) as a marker for a locus does not always lead to a functional basis for a relationship with the phenotype. Direct associations are most convincing when the SNP has a demonstrated functional consequence [29].

Functionality studies

Next to the linking of polymorphisms to phenotypes using association studies, one can investigate the functional consequence of a codon change. *In vitro* studies have shown differences in agonist-promoted downregulation [30], but no

differences were found in agonist binding or agonist-stimulated adenylyl cyclase activities among these polymorphisms of the β_2-AR gene [31]. Differences in adipocyte lipolysis have been reported for this polymorphism [10,32], which might play a role in obesity. It was the first goal for us to investigate the role of the β_2-AR polymorphism in stimulated isolated fat cells. Because of the small number of subjects we were able to include in this study, it was not possible to investigate the genetic role of the β_2-AR gene. Basal lipolysis but not β_2-AR stimulated lipolysis seemed to be somewhat lower in the Arg16Arg group compared to the Gly16Gly group but with 2 subjects in the Arg16Arg group no reliable conclusions could be drawn.

We were able to compare the results of *in vitro* and *in vivo* tests regarding the lipolytic response to β_2-AR stimulation, since some of the subjects participated in both trials. We measured several parameters *in vitro*, like lipolytic capacity and lipolytic sensitivity, and several measures for lipolysis *in vivo*, such as the increase in glycerol and non-esterified fatty acids after stimulation with salbutamol. We found positive relations of lipolytic sensitivity and capacity *in vitro* with lipolytic parameters *in vivo*, although only one of the relations reached statistical significance.

In the *in vivo* study itself, in which the β_2-AR was directly and selectively stimulated with salbutamol, a blunted increase in energy expenditure after stimulation of the β_2-AR in the Arg16Arg subjects was observed. The change in plasma NEFA levels was found to be an important predictor for the change in energy expenditure. In an earlier study of our department Schiffelers et al. [33] showed that obese also had a reduced β_2-AR stimulated increase in energy expenditure and therefore we suggest that this genetic variation might have contributed to this reduced effect. This view is also supported by the association studies described before with a higher prevalence of the Arg16Arg variant in obese subjects compared to lean.

In this *in vivo* study the β_2-AR was selectively stimulated. Selective stimulation does not occur under normal physiological situations and therefore we decided to investigate differences in energy expenditure between polymorphism groups after non selective stimulation of the β-ARs with isoprenaline. During isoprenaline infusion there were no significant differences between the polymorphism groups regarding change in energy expenditure and plasma NEFA concentration. Multiple regression analysis showed that the increase in NEFA and glycerol concentration, and not change

in RER, % body fat, HOMA index or body mass index, was significantly associated with the change in energy expenditure (adjusted for fat free mass). Thus during non-selective stimulation of the β-ARs no differences were found among polymorphism groups.

However, stimulation of the β-AR by intravenous administration of isoprenaline does not reflect a normal physiological response. In real life the physiological agonists of the beta receptors are catecholamines. Therefore with a physiological increase of catecholamines we were able to investigate the role of the β_2-AR gene polymorphism under normal physiological conditions. We used a meal to increase the activity of the sympathetic nervous system [34,35] and consequently to increase plasma catecholamine levels [36,37]. This is in particular true for a high carbohydrate meal, which causes a rapid and large thermogenic response, as was shown before [38]. We were unable to demonstrate an independent contribution of the β_2-AR polymorphism to diet-induced thermogenesis, despite the fact that a significant contribution of the adrenaline response to diet-induced thermogenesis was observed.

The contribution of genetic variation to the development of obesity should be best investigated by challenging the system by overfeeding, which can be considered as the most physiological function test in relation to the obesity problem. A number of overfeeding studies has been described in literature [39-49]. These studies vary in amount of overfeeding, diet composition, and overfeeding duration. Stock [50] analyzed several of these studies and hypothesized that the cost of weight gain is higher on macro-nutrient unbalanced overfeeding diets compared to balanced overfeeding diets. In particular an unbalance in protein is suggested to lead to large variations in weight gain. Dulloo [51] hypothesized that the inter-individual variation in adaptive energy expenditure is relatively small using well-balanced overfeeding diets, whereas overfeeding an unbalanced diet exposes a large range of individual responses. Therefore, to investigate the difference in susceptibility to obesity in humans, different unbalanced diets can be used. We designed a short-term functional test to compare the metabolic responses of a balanced vs. unbalanced overfeeding diet. However, with this protocol we were not able to distinguish subjects susceptible to weight gain from those that were resistant. The low-protein diet showed a lower weight gain compared to the normal diet as expected but no change in energy expenditure could be measured as a consequence of this unbalanced diet. Therefore it was concluded that such short-term overfeeding test to measure the influence of

the β_2-AR polymorphism was not sensitive enough. Dulloo [51] based his hypothesis on results of long-term (3 weeks) overfeeding studies [52,53]. Therefore it seems difficult to design a short-term feasible protocol that can distinguish between subjects susceptible for weight gain and weight stability. Diets with even lower protein content (< 3 Energy%) are too low in protein to use for more than a few days and therefore not acceptable to use as a functional test. Another possibility is to lengthen the overfeeding period to more than 1 week (e.g. 2 weeks). However, this will impose several practical problems which make it not very likely that such a functional test will be applied.

General conclusions which can be drawn from our studies are that we found some functional influence of the β_2-AR polymorphism after direct stimulation of the receptor but when stimulating less selectively the influence of the polymorphism is less pronounced or does not even exist. When the physiological pathway from the receptor downstream to one functional end point is getting longer, more regulatory factors can influence the final outcome and therefore the possible influence of the polymorphism is diluted. However, this does not mean that on the long-term such a very small effect can not contribute to the development of obesity.

The original research design in the light of the final outcome

We originally selected our polymorphism of interest based on the results of a cohort study performed in the Netherlands [9]. In this study β_2-AR polymorphisms seemed to be associated with weight-gain. This relation was different between males and females, where male homozygote subjects (Arg16Arg and Gly16Gly) showed higher weight gain compared to heterozygotes, while in females there was no association. We recruited our subjects from this same cohort [9]. Using this design the idea was to get easily similar group sizes without screening large numbers of subjects before the actual intervention. However, not enough subjects from the cohort were willing to participate in an intervention study and therefore we were not able to select enough subjects to reach sufficient statistical power. The disadvantage of this approach is that subjects initially volunteered to be subject in a cohort and were later asked to participate in intervention studies. Most of the subjects were between 30-50 years old and had a job. They were not willing to take a day off for the interventions.

To study genetic variation in dietary intervention studies a relatively large number of subjects is necessary. However this limits the type and intensity of the interventions. Therefore it would be preferable to start a DNA-data base from all subjects who participate in studies with standard measuring techniques. These techniques can be used for various research questions but the methods are uniform. When consequently DNA samples in all these projects are stored further analysis for polymorphisms is easily possible. At a later stage these SNP results can be associated with intervention parameters that were already measured.
Is this the future of genetic variability research? Or will association studies remain important in this field? Association studies sometimes represent the only practical approach to begin to address a particular biological hypothesis and therefore the number of association analyses will continue to grow, but it's important to recognize their limitations. To reduce the risk of false results, explicit guidelines for genetic association studies may be required. Such guidelines might be, biological rationale, appropriate selection and sampling procedures, rigorous phenotyping and genotyping procedures, large samples, and physiologically meaningful evidence supporting a functional role of the polymorphism [29].

Future directions of genetic research

Despite all genetic research efforts, all associations made between genes and diseases, scientists have to be aware of the ethics of this kind of studies. Can we advise individuals that are more susceptible to a disease to adapt their lifestyle to reduce their increased health risk? But will individuals change their lifestyle? Why do they need the genetic proof to be convinced to change their lifestyle? Will genetic testing not just be another excuse to keep their unhealthy lifestyle? "It's not my fault that I am fat, it's my genes'".
For population scale problems these investigations can be important and also for the increase of knowledge health and disease, but for the individual it could be a problem to see advantages of genetic screening.
In pharmacogenetics research recently the use of polymorphism data to personalize medication did show advantages [54,55]. In anaesthesiology subjects are screened for cholinesterase in their plasma to exclude a genetic deficiency of this enzyme before using succinylcholine as anesthetic. Pharmacogenetics could result in a more restrict and effective use of drugs. For example, the methylenetetrahydrofolate reductase gene polymorphisms showed

relationships with 5-fluorouracil sensitivity [56]. 5-Fluorouracil is used in cancer therapy and difference in sensitivity influences the choice of the medical intervention. This polymorphism has also been linked to adverse effects on plasma homocysteine levels. With an adaptation of the diet or supplementation of folate this adverse effect can easily be solved. Although the dietary effects on weight changes involve a much more complex system, one can speculate about personalized nutrition based on genetic background and susceptibility for obesity. So far, the outcome of the research on linking nutrition to genetic susceptibility in chronic diseases, such as diabetes, obesity and cardiovascular diseases is very disappointing. The originally positive expectation to find the right SNP's causing susceptibility for diseases has been replaced by a realistic scepticism that it will take a long time before personalized diets will become reality in the obesity field.

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Summary

In Western societies the prevalence of obesity has been increasing over the last centuries. As in other diseases, genetic variation influences the prevalence of obesity, in combination with environmental factors. Impairment in energy metabolism leading to changes in energy efficiency can eventually result in obesity. Because of the important role of the sympathethic nervous system (SNS) in the regulation of energy and substrate metabolism, and thus energy balance, genes and proteins involved in this system are targets for genetic research in relation to obesity.
Several association studies showed a relation between genetic variation in the β_2-adrenoceptor gene (at codon 16) and obesity. Therefore, the research presented in this thesis focuses on the functional role of this polymorphism (Gly16Arg variation) and its contribution to the multi-factorial disturbance in energy balance and lipolysis that could be involved in the pathophysiology of obesity.

In the first study (chapter two) we investigated the relationship between body composition and lipolytic activity in vitro after stimulation with the selective β_2-adrenoceptor agonist salbutamol. All correlations between parameters of body composition and lipolysis were negative, but none of them reached statistical significance. In the same experiment we investigated the role of the Gly16Arg β_2-adrenoceptor gene polymorphism in vitro. However, due to the low number of subjects in the study it was difficult to draw conclusions. Some of the subjects participated also in an in vivo experiment (see below). Therefore, we were able to investigate the relation between in vitro and in vivo measurements of lipolytic activity. Few other studies have investigated the relation between in vivo and in vitro lipolytic acivity with inconclusive results. We found a positive correlation between maximal lipolytic rate (in vitro) and salbutamol-induced changes in NEFA (in vivo), indicating a relation between in vivo and in vitro measurements. However, correlations between maximal lipolytic rate and changes in plasma glycerol levels, as well as lipolytic sensitivity and changes in plasma NEFA and glycerol levels, were not found to be statistically significant. The relation between in vivo and in vitro measurements is therefore weak.
In a larger sample of subjects (chapter three) we measured in vivo lipolytic response after selective β_2-adrenoceptor stimulation with salbutamol and compared the different genetic groups for codon 16 of the β_2-adrenoceptor

(Gly16Gly, Gly16Arg, and Arg16Arg). We found a blunted increase in energy expenditure in the Arg16Arg group compared to the Gly-carrier group. In a multiple regression model genetic variation in the β_2-adrenoceptor gene together with the change in plasma NEFA concentration explained 35% of the increase in energy expenditure. These findings might suggest a role for this β_2-adrenoceptor gene polymorphism in the development and maintenance of overweight and obesity.

To further investigate this possible role of the β_2-adrenoceptor gene polymorphism in obesity we investigated the influence of this polymorphism after non-selective stimulation with isoprenaline of the β-adrenoceptor (chapter four), because this would represent a more physiological stimulation than β_2-selective stimulation alone. After non-selective stimulation, changes in energy expenditure were not different among polymorphism groups and the polymorphism in codon 16 did not contribute significantly to the isoprenaline-induced increase in energy expenditure. None of the other measured parameters (change in NEFA, change in glycerol) contributed significantly to the change in energy expenditure, indicating genetic variation in the β_2-adrenoceptor gene and lipolytic activity, are not likely to be the main factors in isoprenaline-induced increase in energy expenditure.

A physiological way to stimulate the β_2-adrenoceptor is with a meal. Diet induced thermogenesis (DIT), and especially the facultative component is mediated by the sympathetic nervous system (SNS). Therefore, in the fourth study (chapter five), we investigated whether the polymorphism at codon 16 in the β_2-adrenoceptor gene would have an influence on DIT. Subjects were fed a high-carbohydrate liquid meal to increase DIT. Increase in plasma adrenaline levels explained 32% of the variation in DIT, where genetic variation in the β_2-adrenoceptor gene did not contribute to the change in energy expenditure. The non-selective stimulation of the β-adrenoceptors after a meal and the difference in adrenaline response to the meal between the groups might have diluted the influence of the genetic variation to DIT.

Because individual differences in weight gain (Up to threefold difference) in response to overfeeding have been described, we investigated in chapter six two short-term overfeeding protocols for their ability to detect metabolic susceptibility to weight gain during overfeeding in humans. With two 7-day overfeeding (+1000kcal/day) periods, different in protein contant (3 energy% protein and 15 energy% protein) we studied the changes in energy expenditure and weight gain. We found a significantly different change in weight between the two diet periods. However, we could not find differences in energy expenditure between the two diets. A small, but not significant,

increase in energy expenditure could not explain the fate of the extra energy intake during low-protein diet. During the normal-protein diet, weight gain completely explained the fate of the excess energy intake. This developed short-term overfeeding protocol was therefore not able to discriminate between individual differences in susceptibility for weight gain during overfeeding.

Overall, the studies in this thesis suggest that there is, at the receptor level (selective stimulation), a functional influence of the β_2-AR polymorphism at codon 16, resulting in a blunted increase in energy expenditure. However, when the stimulation is non-selectively the influence of the polymorphism is less pronounced or does not even exist. When the physiological pathway from the receptor downstream to a functional end point is getting longer and more complex, more regulatory factors will be involved and can influence the final outcome and therefore the potential impact of the polymorphism is diluted. However, this does not mean that on the long-term a very small effect due to a certain polymorphism may not contribute to the development of obesity.

Samenvatting

Het aantal mensen in westerse landen dat te kampen heeft met overgewicht of obees is, is de afgelopen decennia enorm toegenomen. Naast omgevingsfactoren speelt erfelijke aanleg hierbij een belangrijke rol. Een verstoring in energiebalans kan uiteindelijk leiden tot overgewicht. Het sympathisch zenuwstelsel speelt een zeer belangrijke rol bij de regulatie van het energie- en substraatgebruik en dus de energiebalans. Genen die coderen voor eiwitten die een rol spelen in het sympathisch zenuwstelsel zijn daarom interessant voor onderzoek naar genetische aanleg voor obesitas.
Verschillende studies hebben al aangetoond dat er een relatie bestaat tussen genetische variatie in het gen dat codeert voor de β_2-adrenerge receptor (op codon 16) en obesitas. Het onderzoek in dit proefschrift richt zich op de bijdrage van dit polymorfisme aan de regulering van lipolyse en energiegebruik. Verstoringen in deze regulering zouden een rol kunnen spelen bij het ontstaan en het in stand houden van obesitas.
In de eerste studie (hoofdstuk 2) hebben we de relatie tussen lichaamssamenstelling en de afbraak van vet na stimulering met de β_2-adrenerge receptor agonist salbutamol onderzocht in geïsoleerde vetcellen. Alle correlaties tussen parameters van de lichaamssamenstelling en parameters van vetafbraak waren negatief, maar niet statistische significant. Daarnaast werd de bijdrage van variatie in codon 16 van het β_2-adrenerge receptorgen aan de snelheid van de afbraak van vet bestudeerd. Maar door het kleine aantal proefpersonen dat aan deze studie meedeed, waren er geen duidelijke conclusies mogelijk. Bij een deel van de proefpersonen werd de receptor vervolgens ook in vivo gestimuleerd en werd de vetafbraak gemeten. Zodoende was het mogelijk een vergelijking te maken tussen gestimuleerde vetafbraak in geïsoleerde cellen en gestimuleerde vetafbraak in het lichaam. De maximale vetafbraak in geïsoleerde vetcellen was positief gerelateerd met de plasmavetzuurconcentraties na stimulatie. Dit suggereert een relatie tussen in vitro en in vivo metingen van vetafbraak. Echter, correlaties tussen maximale vetafbraak en glycerolconcentraties in het lichaam, alsmede tussen de gevoeligheid van vetafbraak in vitro en veranderingen in vetzuur- en glycerolconcentraties in het plasma in vivo, waren niet significant. Een sterke relatie lijkt dus niet te bestaan.
In een grotere groep proefpersonen (hoofdstuk 3) werd de β_2-adrenerge receptor in vivo gestimuleerd met salbutamol en werd gekeken naar de invloed van genetische variatie (Gly16Gly, Gly16Arg en Arg16Arg) in het gen

voor de β_2-adrenerge receptor op de stijging in het energiegebruik en vetafbraak. De mensen met het Arg16Arg genotype vertoonden minder stijging in het energiegebruik dan dragers van de Gly variant. Samen met de verandering in plasma vrije vetzuren verklaarde de genetische variatie in de β_2-adrenerge receptor 35% van de toename in energiegebruik. Deze bevindingen ondersteunen een mogelijke rol van genetische variatie in de β_2-adrenerge receptor bij de ontwikkeling en het behoud van overgewicht en obesitas.
Om de invloed van deze genetische variatie verder te onderzoeken, werd in een volgende studie niet alleen de β_2-adrenerge receptor, maar werden, met behulp van isoprenaline, ook de andere β-adrenerge receptoren (β_1 en β_3) gestimuleerd. Dit lijkt meer op een natuurlijke fysiologische situatie dan het geval is bij selectieve β_2-stimulatie. Tussen de genetische varianten van de β_2-adrenerge receptor verschilden de stijging in energiegebruik niet significant. Dit laat zien dat de genetische variatie in de β_2-adrenerge receptor geen invloed heeft op de isoprenaline-gestimuleerde stijging in het energiegebruik.
In een vierde studie (hoofdstuk vijf) werd een fysiologische situatie gecreëerd door een hoog-koolhydraat maaltijd aan te bieden en zo het sympathisch zenuwstelsel via de voeding te stimuleren. De facultatieve component van de verhoging van het energiegebruik na een maaltijd wordt gemedieerd door activering van het sympathisch zenuwstelsel. De verandering van plasma adrenalinespiegels na de maaltijd verklaarde 32% van de variatie in energiegebruik. De genetische variatie in het β_2-adrenerge receptoren speelde geen rol van betekenis in de variatie van het energiegebruik. Mogelijk dat de niet-selectieve endogene sympathicusstimulering en de verschillen in adrenalinerespons tussen de genotypes de invloed van de genetische variatie in de receptor heeft teniet gedaan.
Tussen individuen kunnen grote verschillen optreden in gewichtstoename na een overvoedingsperiode. In de laatste studie (hoofdstuk zes) werden proefpersonen op twee verschillende diëten (normaal-eiwit (15 energieprocent) en laag-eiwit (3 energieprocent)) gedurende 7 dagen overvoed (1000 kcal per dag boven de normale inname). Tussen de verschillende diëten was er een significant verschil in de gewichtstoename, waarbij de gewichtstoename op het normaal-eiwit dieet groter was dan op het laag-eiwit dieet. Er werd echter geen verschil gevonden in energiegebruik tussen de twee diëten. Gedurende de laag-eiwit overvoeding kon de kleine, maar niet significante, toename in energiegebruik geen volledige verklaring geven voor het verlies van de extra energie-inname, maar voor de normaal-eiwit overvoeding was alle extra ingenomen energie omgezet in gewicht. Dit korte termijn overvoedingsprotocol is niet in staat gebleken om individuele

verschillen in gevoeligheid voor overgewicht tussen personen aan te tonen. Daarmee was het geen goede functionele test om het verschil in energetische efficiëntie tussen individuen snel te kunnen vaststellen.
Samengevat kan worden gesteld dat de resultaten in dit proefschrift laten zien dat er een invloed kan zijn van het codon 16 polymorfisme in de β_2-adrenerge receptor op op het energiegebruik, wanneer er selectieve stimulering van de β_2-adrenerge receptor plaatsvindt. Echter, wanneer de stimulatie niet selectief is, is de invloed van het polymorfisme minder groot of zelfs afwezig. Naarmate de afstand tussen de receptoren het bestudeerde eindpunt groter is, zijn er meer factoren die invloed kunnen hebben op dit eindpunt en dus het effect van het polymorfisme kunnen verdunnen. Echter dit sluit niet uit dat op lange termijn een klein effect ten gevolge van de aanwezigheid van een bepaald polymorfisme bijdraagt aan de ontwikkeling van overgewicht.

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List of publications

Papers

Oomen, J. and Jeukendrup, A. E. De rol van intramuscular triacylglycerol tijdens inspanning. Geneeskunde en Sport, 31(6): 176-183, 1998

Oomen J.M., C.T.M. van Rossum, W.H.M. Saris and M.A. van Baak. In vitro β_2-adrenoceptor-stimulated lipolysis in relation to body composition and comparison with in vivo stimulation.

Oomen J.M., C.T.M. van Rossum, B. Hoebee, W.H.M. Saris, and M.A. van Baak. β_2-Adrenoceptor polymorphisms and salbutamol-stimulated energy expenditure. Journal of Clinical Endocrinology and Metabolism 2005 Apr;90(4):2301-7

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Oomen J.M., P. Schrauwen, E. Kooi, W.H.M. Saris and M.A. van Baak. Influence of 7 days overfeeding with a low protein or normal protein diet on energy expenditure and weight gain. Submitted

Abstracts

Oomen J.M., W.H.M. Saris and M.A. van Baak. Influence of 7 days overfeeding with a low protein or normal protein diet on energy expenditure and weight gain. *European Congress of Obesity* Helsinki, 2003

Curriculum vitae

Johannes Marinus Oomen was born on the 8th of July 1976, in Breda, The Netherlands. After completing secondary school in 1994 (VWO) at the 'Onze Lieve Vrouwe Lyceum' in Breda, he started the study Health Sciences at the Maastricht University. As part of the specialization for Movement Sciences he started his first research project at Human Biology department on intramyocellular lipid metabolism in trained athlethes during low- and hight fat diet, in 1997. In 1998, within the study specialization of Biological Health Sciences he started a second research project at the Anatomic Institute of the Bern University, Switzerland, on muscular adaptation to high altitude training. In 1999 he received his MSc degree. He directly started working as a PhD-fellow at the department of Human Biology at the Maastricht University. The project entitled 'Genetic variation in energy expenditure', of which the main results presented in this thesis. Currently he is teacher biochemistry at the Zuyd University in Heerlen and member of the Center of Expertise in Life Sciences.